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TWO NEW SPECIES OF THE GENUS *TYPHLODROMUS* SCHEUTEN FROM INDIA (ACARINA: PHYTOSEIIDAE)

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Introduction

Within recent years as a result of the widespread use of organic insecticides, especially chlorinated hydrocarbons, economic entomologists have observed a tremendous increase in the population of phytophagous mites that infest agricultural crops and orchard trees. Indeed, the population is often so large that the mites almost assume the status of a major pest in place of the one that the economic entomologist sought to control by the use of chlorinated hydrocarbons. In this context, the role of predatory mites assumes particular significance. There is also some evidence to indicate that the predatory mites are more susceptible to these insecticides than the phytophagous ones and so with the disappearance of these predatory species in the field that keep the population of the phytophagous mites in a stabilized equilibrium, the latter multiply at a staggering rate and become very destructive to agricultural and horticultural crops.

The mites of the family Phytoseiidæ Berlese (1916) are of great economic importance as members belonging to this family are predators on the phytophagous mites of the families Tetranychidæ, Eriophyidæ and Acaridæ. On account of the importance of this family in the biological control of phytophagous mites, it has attracted the attention of Acarologists all over the world. The taxonomy and biology of these predatory mites are still in a formative stage and though some work has been done in Western countries during the last decade or so, nearly little has been done in India. In biological sciences, it is the experience of workers that in aiming at an enduring contribution on any group, an examination of its taxonomy would naturally engage their first attention. In view of this fact, the authors began a study of the taxonomy of this family sometime ago and the present paper deals with the description of two species that have proved to be new to science. Both of them belong to the subgenus *Amblyseius* Berlese (1914) of the genus *Typhlodromus* Scheuten (1857).

Bernhard (1955) recently revised the family Phytoseiidæ, wherein he has placed the genera *Amblyseius* and *Typhlodromus* as subgenera under genus *Typhlodromus sensu lato*. This has since been supported by Chant (1957) with the remark "The morphological distinctions between the two genera are artificial and such a combination seems logical and acceptable". Evans (1957) has also recognised Bernhard's work in his key to the Phytoseiidæ.

Chætotaxy of the dorsal shield has been used as the basis for this subgeneric separation. But here again confusion has arisen, as various workers (Bernhard, Chant and Evans) have taken different aspects of setal characters into consideration. Bernhard's original classification is based on the number of lateral setæ present on the dorsum between the scapular (S_1) and the lumbar (S_2) setæ. But this system would fail in the case of certain species in which S_2 is absent. So, Chant (1957) devised a more natural system of classification based on the number of anterior lateral setæ on the dorsal shield and the position of S_1 and S_2. This is as follows:—

1. Five or more pairs of anterior lateral setæ present on the dorsal shield; if both S_1 and S_2 present, then more than one lateral seta (L) between their bases .. .. .. Subgenus *Typhlodromus* Scheuten.

Only four pairs of anterior lateral setæ present on dorsal shield; if both S_1 and S_2 present, then only one lateral seta (L) between their bases .. .. Subgenus *Amblyseius* Berlese.

Evans (1957) in his key to the Phytoseiidæ used relative setal length as the basis for his classification which runs as follows:—

1. Setæ L_4, M_2 and L_8 whip-like and considerably longer than the other dorsal setæ .. .. *Amblyseius* Berlese.

Setæ L_4, M_2 and L_8 not conspicuously longer than other dorsal setæ .. .. *Typhlodromus* Scheuten.

But as Chant's (1958) work is more recent, more detailed and more comprehensive and is further based on certain chætotactic features of its immature stages, we are of opinion that his classification should have precedence over those of Bernhard and Evans, and so we have followed his classification in the present paper.

Subgenus AMBLYSEIUS Berlese, 1914

1. *T.* (*A.*) *indicus* sp. n.

Female.—Dorsal shield faintly reticulated and ranging (ten specimens) from 0·325–0·364 mm. in length and from 0·169–0·195 mm. in width,

bearing seventeen pairs of setæ; nine in the lateral (L), two in the median (M), and six in the dorsal (D) rows. Setæ simple and except M_2 and L_6, short and almost equal. Setæ L_1-L_4 less than half as long as distances between their bases [Fig. 1, (1)].

Setæ S_1 and S_2 on interscutal membrane. Peritreme narrow with posterior portion of the shield slightly bulbous at first, tapering to a slender projection that curves around base of Coxa IV. Sternal shield normal, with three pairs of setæ, the third pair being on slight posterolateral projections of the shield. Metasternalia conspicuous, each with a seta. Genital shield normal; ventri-anal shield longer than broad and ranging from ·095–·104 mm. in length and ·074–·082 mm. in width, bearing three pairs of setæ in addition to para-anals and post-anal seta and with a pair of pores slightly posteromediad to third pair of setæ; [Fig. 1 (4)] shield surrounded by four pairs of setæ, one (VL_1) being moderately long, the remainder short. Two pairs of meta-podal plates present, one long and slender almost thrice longer than broad, the other small and ovoid.

Gnathosoma and maxillary palps normal. Fixed digit of chelicera with three teeth and pilus dentilis; movable digit with one weak tooth. Legs normal, with one macroseta (·053 mm.) on basitarsus IV.

Male.—Dorsal shield ranging from 0·234–0·26 mm. in length and 0·140–0·156 mm. in width. Chætotactic pattern of the dorsal shield similar to that of the female except that setæ S_1 and S_2 appear on its edge instead of on the interscutal membrane.

Ventri-anal shield faintly reticulated, 0·106 mm. in length and ·115 in breadth, bearing four pairs of setæ in addition to para-anals and post-anal seta. A pair of pores present as in female [Fig. 1, (5)].

Fixed digit of chelicera 0·023 mm. in length, bidentate, and bearing a strong pilus dentilis. Movable digit 0·019 mm. in length, with one weak tooth and a branched (or T-shaped) spermatophoral process [Fig. 1, (2)].

This species runs close to *T. marinus* (Willman) in having ventri-anal shield longer than broad and setæ L_1, L_2 and L_3 less than half as long as distances between their bases, but differs chiefly in having all setæ (except M_2 and L_6) on the drosal shield equal in length whereas in *T. marinus* the setæ of the dorsal series are noticeably shorter than the lateral. The shape of the 'coxal glands' constitutes a good difference between the two species.

Described from twelve females and four males, collected from "*Gnorimoschema operculella* infested potatoes", feeding on *Tyrophagus putrescentiæ*

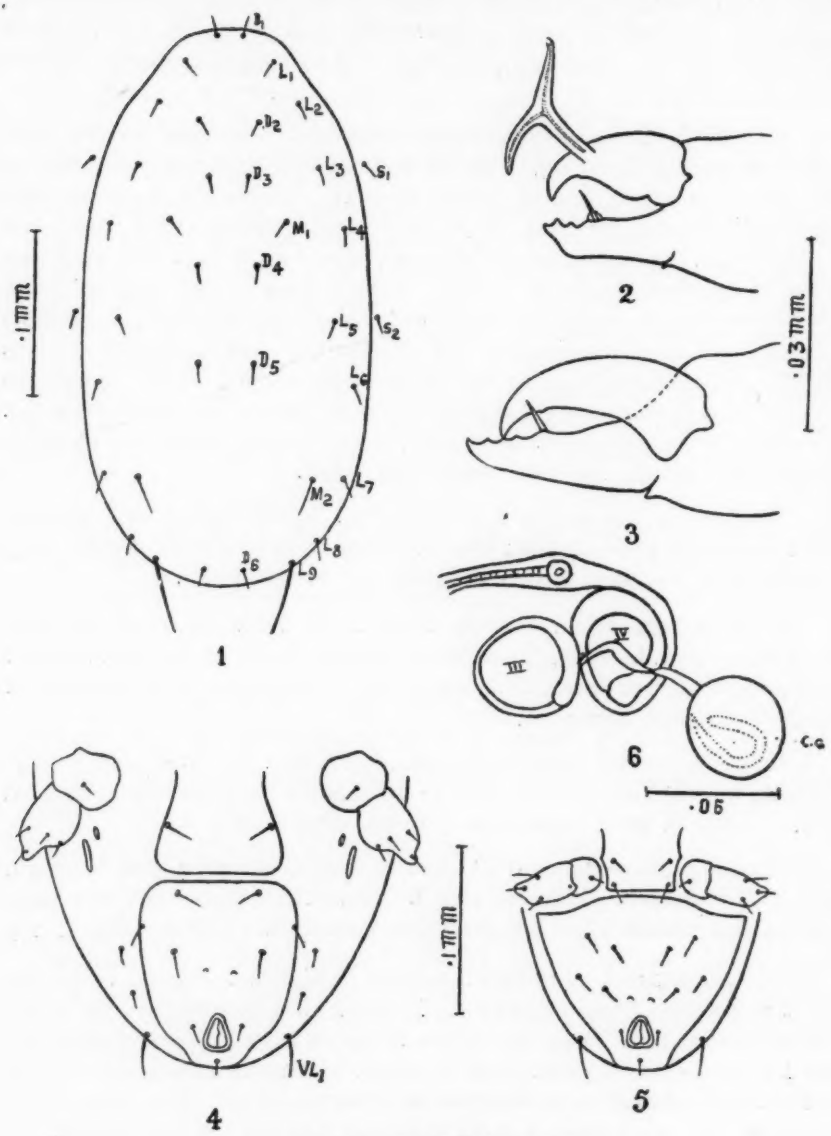

FIG. 1. *T. (A.) indicus* sp. n.

Female: (1) Dorsum; (3) Chelicera; (4) Anal region;
(6) C. G., Coxal gland.

Male: (2) Chelicera; (5) Anal region.

Schrank, at I.A.R.I., New Delhi. Holo-, allo- and para-types, deposited in National Pusa Collection, Indian Agricultural Research Institute, New Delhi.

Coll.—R. B. Kaur; February, 1959.

Tyrophagus putrescentiæ Schrank is a widely distributed mite, which has been found associated with *G. operculella* on stored potatoes, with scales on Sugarcane and *Sorghum* in the field and with fungus growth wherever it is found. The mite, although sometimes suspected to be predaceous, is not actually so, but lives on cast skins and dead pupæ and scales, especially if slightly moist, as it prefers humid media to thrive and develop. It has been observed that if a dry healthy potato is offered to these mites, they do not feed on it, but make only aimless journeys on its surface. This may perhaps be ascribed to the fact that the mites are not able to pierce the skin of a healthy potato for feeding. But if it is tunnelled, and the tunnel after infestation is closed with either a piece of potato or a wet cotton plug, the mites reproduced, multiplied and fed till the tunnel was dried up. This is perhaps the reason for their association with *G. operculella* in cultures. After the insect larva has begun boring into the potato, the mites follow. As the larva goes on boring and feeding, its tunnels are utilized by the mites that take up their abode in their dark, humid and nutrient interior, multiply in millions and exhaust the tuber of its sap.

2. *T.* (*A.*) *delhiensis* sp. n.

Female.—Length 0·35 mm., width 0·195 mm. Dorsal shield smooth, with seventeen pairs of setæ, arranged in a lateral row of nine, a dorsal row of six and a median row of two pairs [Fig. 2, (1)]. All setæ simple except L_9 which is slightly serrated and all setæ big and prominent except D_6. Setæ D_2, D_3 and M_1 comparatively small and of equal length. Setæ L_9 as long as L_4; setæ D_4, D_5, L_5, L_6, L_7, L_8 and M_2 almost equal in length.

Relative lengths of lateral, dorsal and median setæ of the dorsal shield as follows:—

(L_1– L_9) 11 : 8 : 12 : 16 : 9 : 9 : 8 : 9 : 16 : (D_1– D_6) 8 : 4 : 4 : 9 : 9 : 1 : (M_1– M_2 4 : 9.

Setæ M_2 anterior to L_7. All anterior lateral setæ slightly longer than distances between their bases. Bases of setæ M_2, L_7 and L_8 forming a triangle with unequal sides.

S_1 and S_2 on interscutal membrane. Peritreme with the stigmata between coxæ III and IV and running onto dorsum on level of coxa II. Ventral plates not well differentiated. Metasternalia only represented by the setæ.

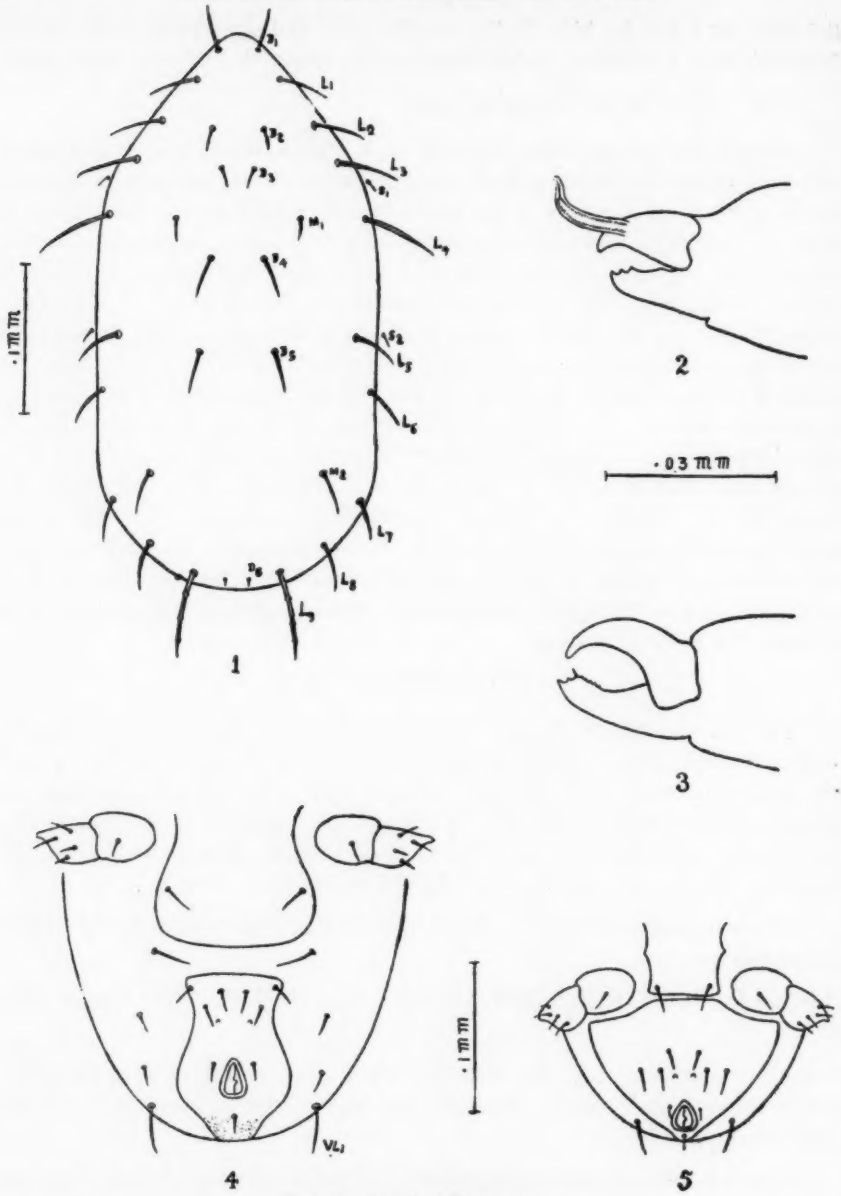

FIG. 2. *T.* (*A.*) *delhiensis* sp. n.

Female: (1) Dorsum; (3) Chelicera; (4) Anal region;
Male: (2) Chelicera ; (5) Anal region.

Ventrianal plate as figured, length 0·098 mm., width 0·054 mm., bearing 3 pairs of setæ in addition to para-anals and post-anal seta. A pair of pores present slightly posterior to the third pair of pre-anal setæ. Shield surrounded by four pairs of setæ, VL_1 being the longest [Fig. 2, (4)].

Gnathosoma and maxillary palps normal. Movable digit of chelicera 0·023 mm. in length, with an extremely weak tooth. Fixed digit 0·026 mm. in length, with two small teeth followed by a number of (about five) minute denticules and with an insignificant pilus dentillis as figured [Fig. 2, (3)].

Legs normal with three macrosetæ on Leg IV, on genu, tibia and basitarsus; the middle one is the smallest.

Male.—Length ranging from 0·25–0·27 mm. and width from 0·15–0·16 mm. Chætotoxy of the dorsal shield as in female except that setæ S_1 and S_2 occur on its edge instead of on the interscutal membrane. This is usual for the mites of this and allied genera (Evans, 1954). Ventri-anal shield as figured, faintly reticulated with three pairs of setæ in addition to pre-anals and post-anal seta. A pair of pores present posterior to the third pair of pre-anal setæ as in female [Fig. 2, (5)].

Fixed digit of chelicera with two prominent teeth (sometimes only one may be seen), pilus dentilis not seen; movable digit toothless and with a spur-shaped spermatophoral process as figured [Fig. 2, (2)].

T. (*A.*) *delhiensis* sp. n. is much closer to *T.* (*A.*) *finlandicus* (Oud.), *T.* (*A.*) *mesembrinus* Dean, *T.* (*A.*) *victoriensis* Womersley and *T.* (*A.*) *ovalis* Evans than to any other published species. It differs from all of these in the relative lengths of the setæ on the dorsal shield. It is also very close to *T.* (*A.*) *aferulus* Chant and *T.* (*A.*) *concordis* Chant (in press) but differs from these in the length of setæ D_4 and D_5. The arrangement of the pre-anal setæ separates *delhiensis* from all species other than those mentioned above; the pre-anal setæ form a nearly transverse row across the anterior ⅓ of the ventri-anal shield.

Described from ten females and five males, collected from Tetranychid infested leaves of *Hibiscus esculentus* H. and *Gossypium* sp. at Indian Agricultural Research Institute, New Delhi. Holo-, allo- and para-types deposited in National Pusa Collection, I.A.R.I., New Delhi.

Coll.—R. B. Kaur; January, 1959.

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* Original not seen.

† It refers to the date of issue of separates of this work and is therefore the date of publication of the new genus.

STUDIES ON THE MICROBIAL SPOILAGE OF CANNED FOOD

II. Effect of Heat, H-ion Concentration and Chemicals on the Spoilage Bacteria

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In the earlier studies (Rangaswami and Venkatesan, 1959), seven bacterial cultures were isolated from spoiled food cans, their harmful effect established, and their systematic position determined. These organisms were found to vary widely in the degree and type of damage caused to the canned food and also in many of their physiological properties. In order to further study their physiological properties and to understand the vulnerable area to combat them the present investigations were undertaken. The H-ion concentration of the food material is known to exert a great influence on the establishment and growth of the contaminant in the cans (Foster and Randall, 1921; Levine and Fellers, 1940; Nolte and Loesecke, 1940; Salle, 1953). Killing the contaminants by heat treatment or creating conditions unfavourable for their growth by the addition of higher concentrations of salt or sugar and other chemical preservatives to food are some of the methods being followed in the canning industry (Wyant and Normington, 1920; Spiegelberg, 1940; Lal and Sadasivan, 1942; Cruess, 1948; Johar, 1951; Williams and Zimmermann, 1951; Baumgartner and Hersom, 1956). The seven bacterial isolates were studied in relation to the above factors and the results are reported here.

Material and Methods

The bacterial isolates, *viz.*, *Bacillus circulans* Jordan, *B. brevis* Migula, *B. subtilis* Cohn, *B. coagulans* Hammer, *B. licheniformis* (Weighmann) Chester, *Clostridium histolyticum* (Weinberg and Seguin) Bergey *et al.* and *Lactobacillus fermenti* Beijerinck obtained in the earlier studies (Rangaswami and Venkatesan, 1959) were used in all these studies.

The thermal death rate of the bacteria was determined by following the method given by Esty and Meyer (1922) and subsequently modified by

Tanner and McCrea (1923). The bacterial suspension was poured in test-tubes and heated in a water-bath for various lengths of time and the surviving bacteria estimated by the dilution plate method. To determine the thermal death point of the bacteria the suspension was heated in a Pressure Heater for one minute at different pressures as recommended by Bigelow and Esty (1920). To determine the thermal death time of the bacteria the suspension was heated in a Pressure Heater for various lengths of time at different pressures. The surviving bacteria were estimated by the dilution plate method and the thermal death time was obtained by plotting the results on a semi-log paper.

To study the effect of H-ion concentration of the medium on bacterial growth, nutrient broth was adjusted to various pH levels with a Beckman pH meter using N/10 citric acid or N/10 KOH solution. The media were distributed in equal quantities into test-tubes, sterilized in the usual manner and inoculated with the bacterial suspension. To another set of the media agar was added and poured in petri dishes after sterilizing. The bacteria were inoculated in the usual manner and their growth examined after three days.

In order to study the effect of salt, sugar and other chemical preservatives on the growth of bacteria, required quantities of the chemical were added to nutrient broth or agar media and tested in the usual manner in test-tubes or in petri dishes. The growth of the test organism was examined after three days.

EXPERIMENTAL RESULTS

Thermal death rate.—The seven bacterial isolates in water suspension were heated at 212° F. in a water-bath. The results obtained are illustrated in Fig. 1.

L. fermenti was found to be least resistant to heat as the cells were killed by a moment's heating at 212° F. Of the spore formers, *Cl. histolyticum* was killed in 90 minutes as compared to 130–400 minutes required for the species of *Bacillus*. The rate of death of the bacterial population also varied with the species; 90 per cent. of the population was killed in three minutes in the case of *Cl. histolyticum*, whereas 24 minutes were required to destroy the same percentage of population in *B. subtilis*.

Thermal death point.—The thermal death points of the seven isolates were determined by treating them for one minute in a Pressure Heater at various pressures ranging from 0–15 lb./sq.in. The estimates of the bacterial population after the treatments are represented in Fig. 2.

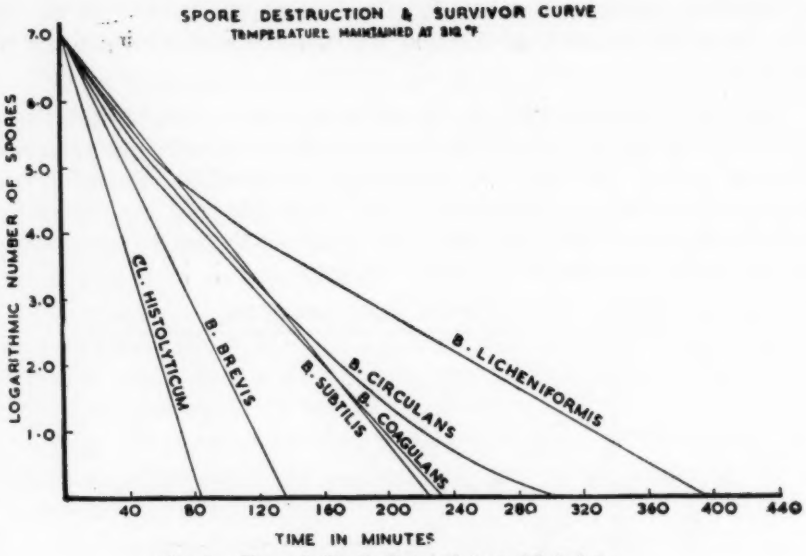

FIG. 1. Thermal Death Rate of bacterial isolates.

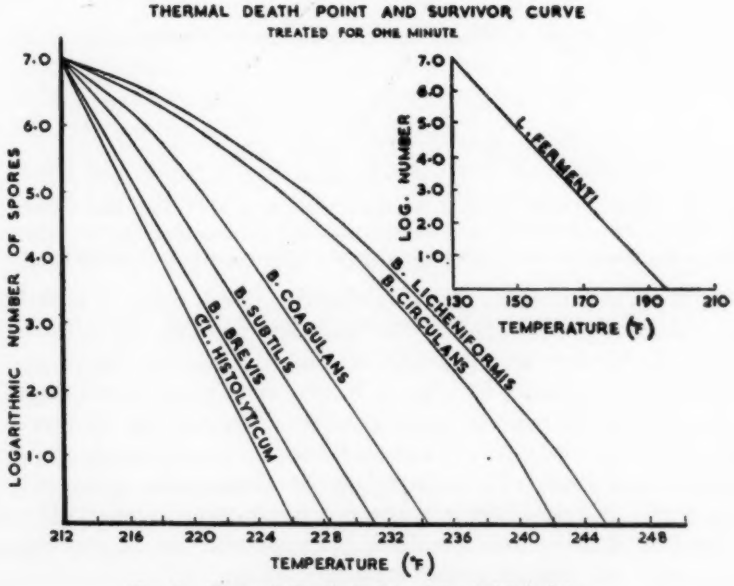

FIG. 2. Thermal Death Point of bacterial isolates.

Again *L. fermenti* was found to be most labile to the treatment as it was killed in one minute at 195° F. The temperature required to kill the other organisms varied from 226–246° F., *B. licheniformis* being the most resistant.

Thermal death time.—The thermal death time for the two of the isolates, *Cl. histolyticum* and *B. licheniformis*, was determined following the procedure described earlier. The time and temperature combinations required to kill the two organisms were determined. The results obtained are represented on a semi-log paper (Fig. 3). The time taken by the bacteria to traverse one log cycle was determined from the graph.

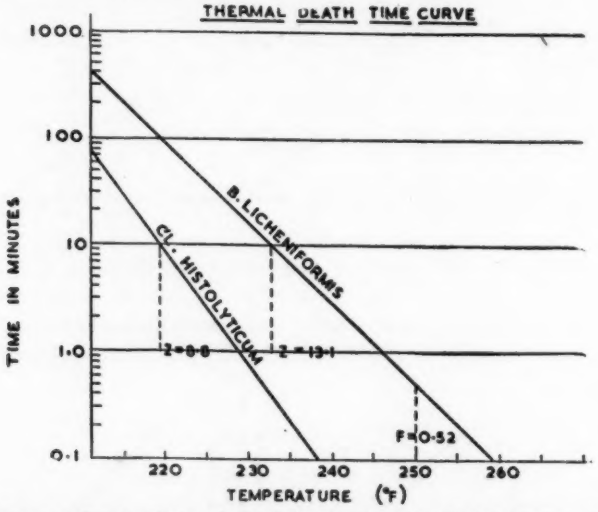

FIG. 3. Thermal Death Time of *B. licheniformis* and *Cl. histolyticum*, represented on a semi-log paper (Z = the slope of the thermal death time curve expressed as ° F. passed over in traversing one log-cycle; F = the number of minutes required to destroy the organism at 250° F.).

From the above results the ' retart heating time ', which is an indication of the resistance put forward by the bacterium to heat, was calculated for *B. licheniformis* by applying the formula given by Stumbo (1948) $U = Z(\log a + p)$, where U = retart heating time for a specific bacterium, Z = the slope of the thermal death time curve expressed as ° F. passed over in traversing one log-cycle, a = initial number of the organism, p = log of the reciprocal of the number of organisms remaining viable at the end of the heating time. The retart heating time (U) for *B. licheniformis* works out to 91·7 minutes, whereas the formula is not applicable to *Cl. histolyticum* as the organism was relatively less resistant to heat.

Effect of H-ion concentration on growth.—The optimum pH levels for the growth of the seven bacterial isolates were determined by growing them in nutrient broth and nutrient agar adjusted to different pH levels.

All the isolates grew in the pH range of 4·5–9·5, but they failed to grow at pH 3·0 except *L. fermenti*, which was found to grow well at that pH. The optimum pH for the spore formers was found to be between 5·5–8·5 and for *L. fermenti* it was between 3·5–8·0.

Salt tolerance.—The salt tolerance capacities of the bacterial isolates were tested in nutrient broth and in nutrient agar media containing different concentrations of sodium chloride.

L. fermenti did not multiply even at 4 per cent. salt concentration, and *Cl. histolyticum* and *B. brevis* at 6 per cent. concentration, whereas the other organisms could tolerate even higher concentrations, *B. licheniformis* being capable of growing at 13 per cent. concentration.

Sugar tolerance.—The sugar tolerance capacities of the bacteria were examined in nutrient broth and nutrient agar media containing different concentrations of cane sugar.

The results indicate, that in general, 40–45° B. is required to inhibit the bacterial growth, but *Cl. histolyticum* was relatively less tolerant as it failed to grow even at 30° B.

Effect of chemical preservatives on the bacteria.—Two chemical preservatives, *viz.*, Na-benzoate and potassium meta-bisulphite (K.M.S.), commonly used in the industry, were tested for their capacity to inhibit the bacterial isolates. The results are summarized in Table I.

It was found that 1,300 p.p.m. of Na-benzoate or 450 p.p.m. of K.M.S. are required to completely inhibit all the bateria tested, but 700 p.p.m. of the former or 150 p.p.m. of the latter is sufficient to reduce the growth of the bacteria. Of the seven isolates, *L. fermenti* was most susceptible to both the chemicals, *B. subtilis* and *B. coagulans* most resistant to Na-benzoate and *B. circulans* and *B. subtilis* most resistant to K.M.S.

Influence of H-ion concentration and sugar content on the action of chemical preservatives.—In order to study the effect of the two chemicals, alone and in combination with each other, on the bacteria, as influenced by the H-ion concentration and sugar content of the substratum, another experiment was conducted. *Cl. histolyticum*, *L. fermenti* and *B. licheniformis*, belonging to three different genera, were selected for the purpose. Five hundred p.p.m. of Na-benzoate and 200 p.p.m. of K.M.S., the concentrations found in the previous experiment to be inhibitory to the three organisms,

TABLE I

Inhibitory action of chemical preservatives on bacterial isolates

(Tested in nutrient agar medium for three days)

Bacterial culture	Inhibitory conc.: p.p.m.	
	Na-benzoate	Potassium meta-bisulphite
B. circulans ..	1,000	500
B. brevis ..	900	400
B. subtilis ..	1,300	500
L. fermenti ..	700	300
Cl. histolyticum ..	1,000	400
B. coagulans ..	1,300	400
B. licheniformis ..	1,200	400

and a combination of half the strength of the two substances were tested on the bacteria in nutrient broth and nutrient agar media containing different concentrations of sugar and adjusted to various pH levels. The minimal inhibitory concentration of sugar at various pH levels was obtained and the results are represented in Fig. 4.

It was found that the chemicals were effective in acid and alkaline ranges than at neutral range. Also when the chemicals were tested together at half the concentrations, they were more effective indicating thereby a synergestic action on the bacteria. Also when the chemicals were tested at various sugar concentrations it was found that the bacteria were completely inhibited at lower pH and sugar combinations.

DISCUSSION

It is well known that the spore-forming bacteria withstand adverse environmental conditions, especially heat, better than others. Though this was found to be true in the present investigations, wide ranges in the heat resistance and chemical tolerance qualities were found among the species. Reynolds and Lichtenstein (1952) analysed the data on heat destruction of

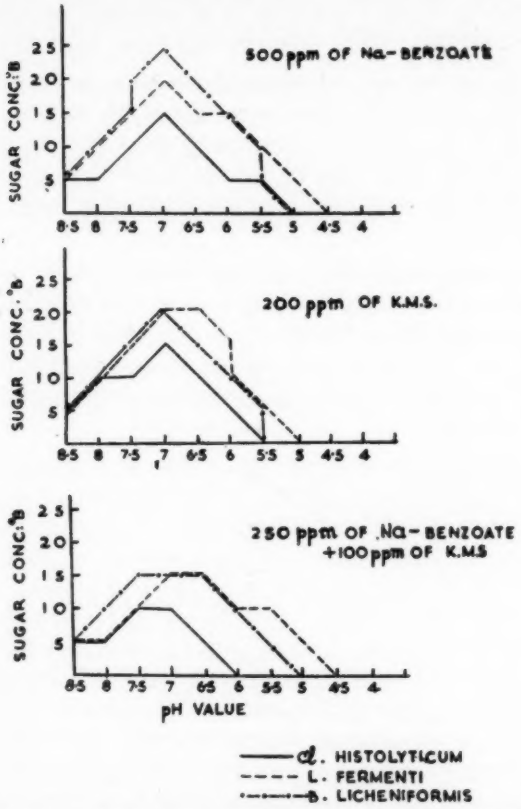

FIG. 4. The influence of H-ion concentration and sugar content of the medium on the action of Na-benzoate and potassium meta-bisulphite (K.M.S.), alone and in combination, on three bacterial species.

many spore-forming bacteria and differentiated three phases in their death rate; an initial phase of accelerated death, a second phase of logarithmic destruction and a final phase of decreasing death rate. The rate of destruction of the spores of *B. licheniformis*, *B. circulans* and *B. coagulans* seem to follow a similar pattern but in *L. fermenti*, *Cl. histolyticum* and *B. brevis* there were no distinct phases of death rates.

The results obtained in the studies on the pH relationships indicate that all the seven bacterial contaminants are capable of causing damage to canned food in the pH range of 4·0–9·0. Most of the vegetable products including tomato and fruit juices have a pH range between 4·0–6·0 and so are capable

of multiplying even at pH 3·0 and so is more dangerous than the rest as it can destroy even highly acidic products like citrus juice and pickles.

The studies on the salt tolerance of the bacteria have clearly brought out that *L. fermenti* and *Cl. histolyticum* are highly sensitive to salt and so can be checked in the industry by increasing the salt percentage of the food medium wherever possible. But *B. licheniformis* is capable of living in high salt concentrations and so is to be carefully watched in foods containing high salt concentrations.

Regarding sugar tolerance, all the seven isolates are inhibited at 40–45° B.; but this concentration is much lower than those required to inhibit yeasts and moulds (Tanner, 1944). *Cl. histolyticum* seems to be easily inhibited by sugar as it failed to grow at 30° B. and so this property can be advantageously utilized to check the spreading of the organism in the food preservation industry.

The two chemical preservatives tested showed that they act in a synergestic manner when combined together as they are much effective in checking the organisms than when tested individually. The influence of sugar content and H-ion range on their effectiveness also indicates very encouraging results. Though more detailed investigations are needed to understand the reasons for the synergestic effect and for the influence of H-ion and sugar contents on the effectiveness of chemicals, the results obtained here indicate that when once the pH of the food material to be preserved is known, a combination of the two chemicals together with calculated quantities of sugar can be used to check the microbial spoilage. Other chemical preservatives have been recently tested in India on spoilage moulds of mango squash (Anand *et al.*, 1958). Work in this line to test various chemical preservatives on the bacterial contaminants in a given set-up will be of much value.

SUMMARY

1. Seven bacterial species, *viz.*, *Bacillus circulans* Jordan, *B. brevis* Migula, *B. subtilis* Cohn, *B. coagulans* Hammer, *B. licheniformis* (Weighman) Chester, *Clostridium histolyticum* (Weinberg and Seguin) Bergey *et al.* and *Lactobacillus fermenti* Beijerinck, isolated earlier from spoiled canned foods, were studied for their physiological properties in relation to heat treatment, H-ion concentration of the substratum and susceptibility to chemical preservatives.

2. The species of *Bacillus* were highly resistant to heat treatment, *Cl. histolyticum* less resistant and *L. fermenti* labile to heat. The rate of death of the bacterial population in *B. licheniformis*, *B. circulans* and *B. coa-*

gulans followed three distinct phases, firstly a major population was destroyed in a relatively short period, followed by a rapid logarithmic destruction and finally a decreasing rate of death. In *B. brevis*, *B. subtilis*, *Cl. histolyticum* and *L. fermenti* the population was destroyed in an almost uniform pattern.

3. All the seven bacterial species were found to thrive well in a wide range of pH and *L. fermenti* was capable of multiplying even at pH 3·0 indicating that it can spoil highly acidic food products.

4. *L. fermenti* and *Cl. histolyticum* were highly sensitive to salt concentration of the medium, whereas *B. licheniformis* was found to be a facultative halophile as it could grow even in 13 per cent. salt concentration.

5. In general all the bacteria were inhibited at a sugar concentration of 40–45° B., but *Cl. histolyticum* was relatively less tolerant as it failed to grow even at 30° B.

6. Na-benzoate and potassium meta-bisulphite, when tested on the bacteria, were found to have a synergestic effect when combined together than when tested alone. They were more effective in acid and alkaline ranges of the substratum than in neutral range. Also, when the combination of the chemicals was tested at various sugar concentrations of the medium, only half the concentration of the sugar could suffice to inhibit the bacteria.

7. On the basis of the results it is suggested that *L. fermenti* could be easily eliminated by heat treatment or addition of salt to the product, *Cl. histolyticum* by heat, or addition of salt or sugar and the species of *Bacillus* by a combination of Na-benzoate and potassium meta-bisulphite in a sugar medium acidified with edible acids.

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STUDIES IN THE *CAESALPINIACEAE*

III. Cytological Observations in *Cassia* Species

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Introduction

Cassia Linn. is a large genus, comprising approximately 400 species distributed in the warm regions of both the Worlds. Taxonomists like Moench, Meyer, Kunth and others (*see* Senn, 1938 *b*) proposed that it should be split up into two genera, namely, *Cassia* and *Chamæcrista*. *Cassias* show great variation in habit ranging in size from large trees to small annual herbs. Considering the large size, taxonomic problems and the wide range in the habit in the genus, cytology seems to have not received adequate attention. Cytological work in the genus has so far been mainly limited to records of chromosome numbers and observations on the somatic mitosis in some species (Jacob, 1940). In the present paper results of a study of the meiosis in nine species, all of which grow wild or in a cultivated state in India, are presented and their bearing on the cytotaxonomy of the genus has been examined.

Previous Work

The earlier cytological work in this genus which was largely restricted to chromosome number reports only was summarised by Senn (1938 *a*) and Jacob (1940). The important recent works dealing with cytotaxonomic and cytological are those of Senn (1938 *a* and *b*) and Jacob (1940).

Senn (1938 *a*) recorded chromosome numbers in *C. tora* ($n = 13$), *C. chamæcrista* ($n = 8$), *C. nictitans* ($n = 8$), *C. alata* ($n = 6$) and *C. occidentalis* ($n = 14$). In *C. nictitans* he observed somatic doubling in root tip cells. In another paper (Senn, 1938 *b*) he discussed the taxonomic problem taking the cytological data into consideration. On the basis of chromosome numbers he supported the splitting of the genus into two genera *Cassia* and *Chamæcrista*.

Jacob (1940) studied the relationship of sat-chromosomes to the nucleoli in *C. auriculata*, *C. tora*, *C. Javanica*, *C. grandis*, *C. floribunda*, *C. corym-*

bosa and *C. siamea*. In all these except the first species he found the diploid number to be 28 and showed the previous reports for *C. tora* $n = 13$ by Datta (1935) and Senn (1938 *a*) were incorrect. In *C. auriculata* there are two types according to him, one with $2n = 14$ and another with $2n = 16$ chromosomes. In all the species of *Cassia* studied by him except *C. corymbosa* and *C. siamea* he observed 2 nucleoli formed at telophase and 2 sat-chromosomes taking part in the organization of the nucleoli. In *C. siamea* 4 sat-chromosomes were observed and 4 nucleoli organized at telophase. In *C. corymbosa* also there is the formation of 4 nucleoli at telophase.

The present writer (Pantulu, 1940, 1942, 1946 *a* and *b*) has reported the chromosome numbers in *C. auriculata* ($n = 14$), *C. glauca* ($n = 14$), *C. glauca* var. *suffruticosa* ($n = 28$), *C. marginata* ($n = 14$), *C. obtusifolia* ($n = 13$), *C. siamea* ($n = 14$), *C. sophera* ($n = 14$) and *C. fistula* ($n = 14$).

More recently Atchison (1951) and Turner (1956) recorded the chromosome numbers for several species of *Cassia*. Atchison (1951) in addition to other plants belonging to Leguminosæ studied the chromosome numbers in the following species of *Cassia*. *C. fastuosa* ($2n = 24$), *C. moschata* ($2n = 28$), *C. nodosa* ($2n = 24$), *C. renigera* ($2n = 24$), *C. antiflaner* ($2n = 24$) and *C. atomarica* ($2n = 24$). Turner (1956) noted the chromosome numbers in 4 species; namely, *Cassia durangensis* ($n = 14$), *C. fasciculata* var. *feniscai* ($n = 8$), *C. occidentalis* ($n = 14$) and *C. rœmeriana* ($n = 14$).

MATERIALS AND METHODS

The present investigation deals with the following species of *Cassia*:—

(1) *C. glauca* Lam.

(2) *C. glauca* var. *suffruticosa* koenig.

(3) *C. occidentalis* L.

(4) *C. Sophera* L.

(5) *C. auriculata L.*

(6) *C. marginata* Roxb.

(7) *C. obtusifolia* L.

(8) *C. fistula* L. and

(9) *C. alata* L.

The materials were fixed in Nawashin's fixative between 11 A.M. and 1 P.M. Dehydration and embedding in paraffin was carried out according to the customary methods. Sections were cut at 10–14 μ thick and were

stained with Newton's iodine crystal violet. One per cent. chromic acid was used as a mordent either before or after the stain.

In addition to the sections, permanent as well as temporary aceto-carmine smears of pollen mother cells were studied in *C. occidentalis*, *C. sophera*, *C. auriculata*, *C. alata* and *C. fistula*.

Observations

C. glauca Lam.

At diakinesis stage, in this species, a single nucleolus was observed and two bivalents were in association with it (Fig. 1). One of the bivalents very faintly shows the satellited nature of the chromosomes. In the polar view of metaphase, 14 bivalents can be clearly seen (Figs. 2–4). The bivalents at this stage arranged on the equatorial plate exhibit clear secondary pairing. Such pairing first appears at the metaphase I, persists to some extent through the anaphase and reappears prominently again during the metaphase II (Figs. 5).

The number of bivalents exhibiting secondary pairing was observed to vary. The maximum associations noted were 2 (3), 3 (2), 2 (1) or 1 (3), 5 (2), 1 (1), thus giving a total of 7 associations. The maximum association was observed in 4 cases out of 28 metaphase plates examined.

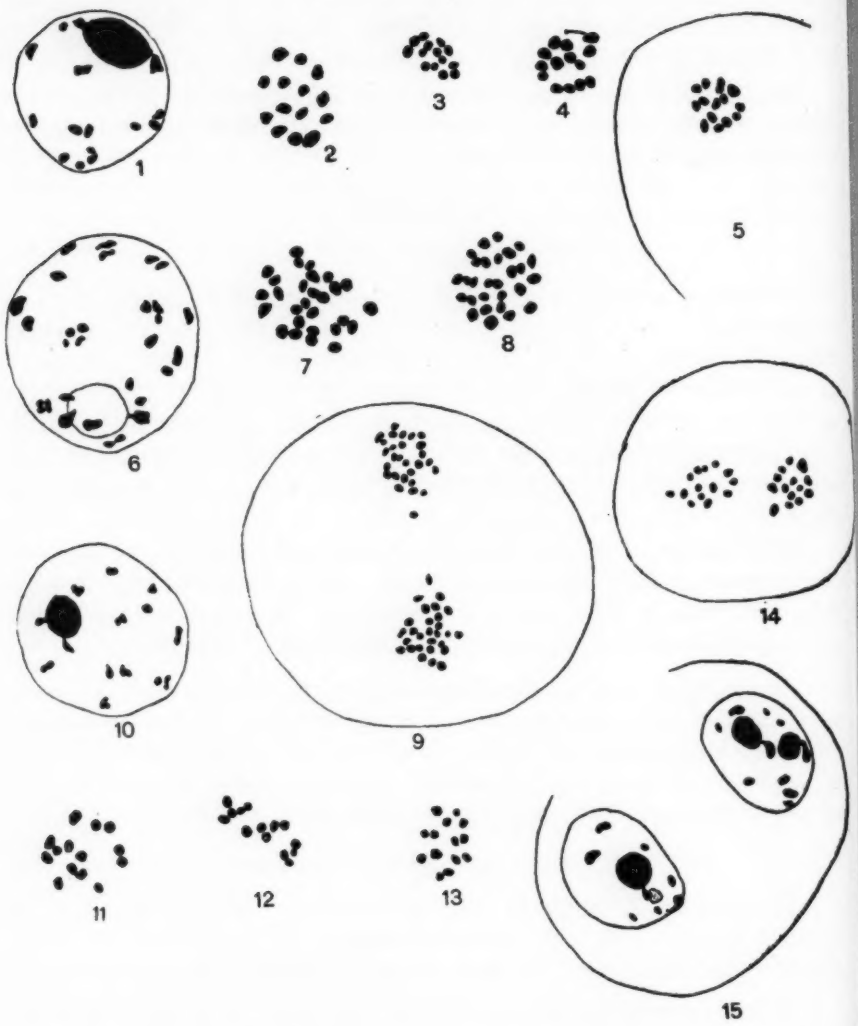
At anaphase there is normal separation of the chromosomes to the two poles, and the chromosomes after reaching the poles organize themselves into interkinetic nuclei. In the organization of the nucleolus, first a number of bodies are formed. Later these fuse together and give rise either to one or two nucleoli as observed by Jacob (1940) in *C. grandis*.

C. glauca var. *suffruticosa* koenig.

In this form 28 bivalents were clearly seen at the metaphase stage (Figs. 7 and 8). This is the highest number so far recorded in the genus. All other species now studied show a uniform number $14n$ chromosomes.

There is a single nucleolus at the diakinesis stage and in association with it are 4 bivalents (Fig. 6). At metaphase stage here also secondary pairing was observed and a variable number of bivalents were observed to enter into association (Figs. 7 and 8).

The anaphase separation of the chromosomes was normal and at the metaphase II 28 chromosomes were again clearly counted (Fig. 9). At telophase I at first 4 nucleoli were noticed these later fuse and give rise to a single large nucleolus.



TEXT-FIGS. 1–15. Figs. 1–5. *Cassia glauca*. Fig. 1. Diakinesis showing 2 bivalents in association with the nucleolus. Fig. 2. Metaphase I showing clearly 14 bivalents. Figs. 3 and 4. Metaphase I showing secondary association. Fig. 5. Metaphase II. Figs. 6–9. *Cassia glauca* var. *suffruticosa*. Fig. 6. Diakinesis showing 4 bivalents in association with the nucleolus. Figs. 7 and 8. Polar view of Metaphase I showing 28 bivalents and various degrees of secondary association. Fig. 9. Metaphase II. Polar views showing 28 chromosomes. Figs. 10–15. *Cassia occidentalis*. Fig. 10. Diakinesis stage showing 2 bivalents in association with the nucleolus. Figs. 11–13. Representative Metaphase I plates showing various degrees of secondary association. Fig. 14. Metaphase II. Fig. 15. Telophase I one of the nuclei having two nucleoli and one chromosome in association with each nucleolus. All Figures, ×1, 250.

C. occidentalis L.

Out of the two bivalents associated with the nucleolus at the diakinesis stage, one shows clearly the satellited nature of the chromosomes. In polar views of metaphase plates, 14 bivalents were clearly observed (Figs. 11–13). Previously for this species, Muto (1929) reported the haploid chromosome number as 13. This number is incorrect not only according to the writer but also according to Senn (1938 *a*) who also counted 14 *n* chromosomes. The number $n = 13$ is rather unusual for any species in this genus. In *C. tora* also where this number had been reported by Datta (1935) and Senn (1938 *a*) Jacob (1940) has proved it to be incorrect. He observed 28 chromosomes in the root tip cells.

At the metaphase I, studied from sectioned material as well as smears, secondary association was prominently noticed. The maximum association of 7 was observed in 10 out of 26 cases studied (Figs. 11–13).

There is normal separation of the chromosomes at anaphase, and in the polar views of metaphase II 14 chromosomes were clearly seen (Fig. 14). The organisation of the nucleolus at the telophase I is as in *C. glauca*, *i.e.*, 4–6 small bodies of irregular shape appear at first and these later fuse to give rise to either one or two nucleoli. In association with each nucleolus, when two are present, is one chromosome (Fig. 15).

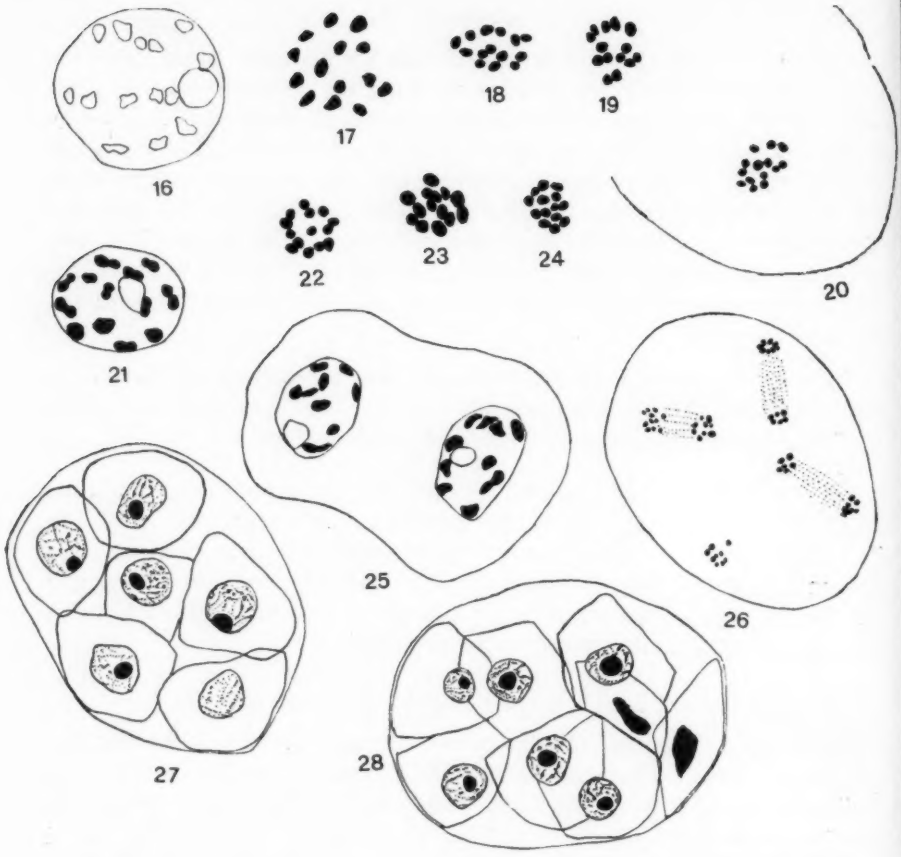
C. sophera L. (*C. purpurea* Roxb., *C. torosa* Cav.)

Previously Kawakami (1930) reported the chromosome number for this species as $n = 12$. Ghose and Alagh (1933) working on this species under a different name *C. purpurea* Roxb. reported the chromosome number as $n = 10$. Both these reports appear to be incorrect. On the other hand Nakajima (1936) (quoted by Senn, 1938) working on this species as *C. torosa* Cav. reported the chromosome number as 14 *n*, and it agrees with the present observations (Fig. 17). This shows that for *Cassia* species any number other than a multiple of 7 should be accepted with caution.

At diakinesis here also two bivalents were observed to be in association with the nucleolus (Fig. 16). Secondary association was observed in this species also at metaphase I as well as metaphase II. The maximum association of 7 was noted in 9 cases out of 24 studied (Figs. 18 and 19).

C. auriculata L.

This is an interesting example of a plant showing aneuploidy as well as polyploidy within the same species. Jacob (1940) worked on plants



TEXT-FIGS. 16–28. Figs. 16–20. *Cassia sophera*. Fig. 16. Diakinesis stage showing 2 bivalents in association with the nucleolus. Fig. 17. Metaphase I from temporary acetocarmine smear preparation. Figs. 18 and 19. Metaphase I plates showing secondary association. Fig. 20. Metaphase II. Figs. 21–28. *Cassia auriculata*. Fig. 21. Late diakinesis. Figs. 22–24. Metaphase I plates showing various degrees of secondary association. Fig. 25. Two-nucleate pollen mother cell. Fig. 26. Two-nucleate pollen mother cell with both nuclei dividing to give rise to 8 microspores. Fig. 27. Pollen mother cell with six microspores. Fig. 28 with 8 microspores. All Figures. except Figs. 27 and 28, ×1,250. Figs. 27 and 28, ×600.

grown from the seeds collected from Ceylon and he observed two chromosomal types one with $n = 7$ and another with $n = 8$. In both the cases there are 2 sat-chromosomes, and 2 nucleoli are formed at mitotic telophase. The present writer studying the material from Waltair reported $n = 14$ (Pantulu, 1940). In the present paper in addition to Waltair material,

material collected from Guntur also was studied and in both cases the same number, *i.e.*, $n = 14$ was observed. Thus the plants having more northern distribution are tetraploids when compared to the Ceylon plants.

At diakinesis stage one nucleolus was observed and 2 bivalents in association with it (Fig. 21); and this is in conformity with Jacob's observations. Jacob (1940) observed one pair of sat-chromosomes in the root-tip cells and that is a diploid plant. The present material shows 2 bivalents in association with the nucleolus at diakinesis stage and may be considered to be a tetraploid.

In the material collected from Waltair a number of binucleate pollen mother cells were observed. This condition seems to be due to the non-formation of the walls at the end of the premeiotic mitosis. Due to lack of space there is crowding of the P.M.C.s and the two cells without a separating wall remain together as a single cell with two nuclei (Fig. 25). The division of these nuclei is quite normal and ultimately each of these bi-nucleate P.M.C. gives rise to 8 microspores (Figs. 26–28) which however, become non-functional and degenerate.

C. marginata Roxb.

Only a few flower buds of this material were available and all these were in advanced stages and showed only meiosis II. At metaphase II 14 chromosomes were clearly counted (Figs. 29–30). Secondary association was also clear at this stage and the maximum association of 7 was observed (Figs. 29 and 30). The nuclei of Telophase I have either one or two nucleoli; when there is a single nucleolus 2 chromosomes were found to be taking part in the organization (Fig. 31). The organization of the nucleolus here resembles that of *C. glauca* and *C. occidentalis.*

C. obtusifolia L.

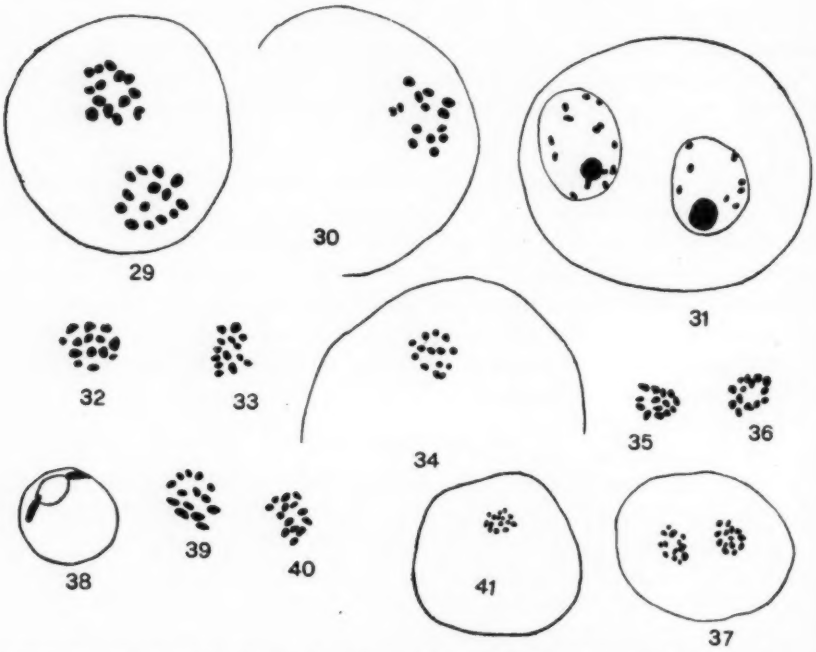
Previously for this species $n = 13$ chromosomes was reported by the writer (Pantulu, 1942) but further study has revealed that the previous count was incorrect and this species also has $n = 14$ chromosomes (Figs. 32–34).

At diakinesis stage there is a single nucleolus and in association with it there is a single bivalent; Jacob (1940), in the closely related *C. tora*, observed in the root-tip cells 2 nucleoli at telophase, and that the sat-chromosomes could not be made out because of the small size of the chromosomes.

C. fistula L.

According to the international rules of botanical nomenclature, 1935, it has been proposed that *Cassia fistula* Linn. should be selected as the type

species of the genus *Cassia*. This species belongs to the sub-genus *Cathartocarpus* Pers. Tischler (1921–22) has recorded the chromosome number of this species as $n = 12$. This number is unusual in this sub-genus. A large



TEXT-FIGS. 29–41. Figs. 29–31. *Cassia marginata*. Figs. 29 and 30. Polar views of Metaphase II. Fig. 31. Telophase I; one of the daughter nuclei having a single nucleolus and in association with it 2 chromosomes. Figs. 32–34. *Cassia obtusifolia*. Figs. 32 and 33. Polar views of Metaphase I showing 14 bivalents. Fig. 34. Polar view of Metaphase II showing 14 chromo somes. Figs. 35–37. *Cassia fistula*. Figs. 35 and 36. Metaphase I. Fig. 37. Metaphase II. Figs. 38–41. *Cassia alata*. Fig. 38. Diakinesis stage showing 2 bivalents in association with the nucleolus. Figs. 39 and 40. Polar views of Metaphase I showing 14 bivalents. Fig. 41. Polar view of Metaphase II showing 14 chromosomes. Figs. 29–31, ×1,250; All the rest, ×650.

number of counts made both at metaphase I and II revealed the chromosome number $n = 14$ (Figs. 35–37) (also Pantulu, 1946). Thus the observations of the writer are in variance with Tischler's report; but are in conformity with the chromosome numbers so far reported for a large number of species of *Cassia* which do not fall under the section *Chamæcristæ*.

In this species also secondary association was examined and a maximum association of 7 was observed.

C. alata L.

Senn (1938) recorded the chromosome number of this species as $2n = 12$ from the root-tip cells. This number $2n = 12$ is unusual in the genus where there are two polyploid series one with the basic number 7, and the other with the basic number 8. In the present investigation a large number of counts made from smear preparations of P.M.C. and sectioned material (of *C. alata*) revealed the chromosome number as $n = 14$ (Figs. 39–41). This number differs from that reported by Senn (1938).

At diakinesis stage 2 bivalents were observed to be in association with the nucleolus (Fig. 38). Senn (1938) has noted 2 sat-chromosomes in the diploid complement of 12 chromosomes.

At metaphase I the maximum association of bivalents into 7 groups was observed. The anaphase separation is normal and at telophase I, 2-nucleoli are organized at first.

Discussion

Polyploidy

Polyploidy within the species is found in *C. auriculata* and *C. alata* among the species included in the present investigation. The material of *C. auriculata* collected from the northern parts of the Indian Peninsula shows $n = 14$ chromosomes, whereas the material from Ceylon worked out by Jacob (1940) shows $n = 7$ chromosomes. *C. alata* collected from cultivated plants in India shows $n = 14$ chromosomes, while the American material worked out by Senn (1938 *a*) shows $n = 6$ chromosomes. Thus both the materials investigated here are presumably tetraploids. Previously polyploidy within the species was noted in a number of cases (*cf.* list of Darlington, 1937). In the genus *Cassia* diploid and tetraploid forms were recorded by Kawakami (1930) in *C. mimosoides*.

According to Darlington (1937) the polyploid forms within the species may differ in size and other morphological characters from their diploids, but in some cases the diploid and polyploid forms are indistinguishable from each other although they differ in their distribution. The tetraploid forms of *C. auriculata* and *C. alata* belong to the latter category. In these a good seed-set takes place and the cytological behaviour resembles that of allopolyploids in the absence of multivalent formation at metaphase. It is possible that polyploids within these species might have originated as autopolyploids and subsequently differentiation or structural changes in the chromosomes such as suggested by Jacob (1940) in *C. auriculata* might have

taken place ultimately resulting in the cytological behaviour characteristic of allopolyploids (Darlington, 1937 and Stebbins, 1947).

C. glauca var. *suffruticosa* koenig. with the highest chromosome number ($n = 28$) so far recorded in this genus is an octoploid. With the available data it is, however, not possible to speculate on the probable origin of this variety. It is interesting to note that certain taxonomists (*cf.* Gamble, 1915) have raised this variety to the rank of a species (*C. suffruticosa* W.A.) on the basis of morphological characters. This is supported by the evidence of chromosome numbers. All the other species studied show a uniform chromosome number $n = 14$.

Chromosome numbers and growth habit.—In the genus *Cassia* the species vary in their growth habit from small annual herbs like *C. obstusifolia*, *C. tora* to large trees as *C. javanica*, *C. siamea* and others. In all these species so far studied there is a uniform chromosome number of $n = 14$ and even in the chromosome size between the herbs and the trees no marked differences were observed.

Stebbins (1938 and 1947) tried to correlate the chromosome numbers and growth habits in the dicotyledons. When he compared the chromosome numbers of 205 genera of woody dicotyledons, 151 genera of herbaceous dicotyledons and 22 genera containing both woody and herbaceous species he observed that the woody plants in general have a higher basic number than the herbaceous ones. This fact according to him strongly suggested the polyploid origin of woody plants. But this hypothesis was not supported when comparisons were made between woody and hebraceous species within the same genus or family. In all these genera where both woody and herbaceous species are present, the woody species are not predominantly polyploid. On the other hand in some genera all or nearly all the polyploid species are herbaceous.

Senn (1938) from his extensive studies on the chromosome numbers in the *Leguminosæ* arrived at the conclusion that the perennial species have arisen from annual types with lower chromosome numbers and that the woody legumes may have originated from herbaceous ones with lower basic number.

Thus there seems to be no consistent correlation between the chromosome numbers and growth habits. In the genus *Cassia* when there are woody as well as herbaceous species with uniform chromosome numbers, the chromosome numbers do not give any clue to the problem.

Taxonomic Problem

The section *Chamæcrista* under the genus *Cassia* was first created by Linnaeus in 1753 consisting of five species. From that time on this section had almost a rambling taxonomic history. Some taxonomists raised it to the status of a genus; some others kept it as a section and still others amalgamated it with *Cassia* (Senn, 1938 *b*).

Senn (1938 *b*) tried to examine this problem from the cytological point. He states that in the *Leguminosæ* aneuploidy frequently is involved in the generic differences and he points out the species that are included under the section *Chamæcristæ veræ* Benth. showed an orthoploid series 8, 16, 24 whereas the other numbers 6, 13, 14 are distributed throughout the sub-genus *Cathartocarpus* Pers. and the section *Oncolobium* vog., *Prososperma* vog. and *Chamæsenna* D.C. of the sub-genus *Senna* Roxb. Benth. On the basis of these chromosome numbers he tentatively suggests that " the group of species comprising the polyploid series with the basic number 8 falls within the limits of the genus *Chamæcrista* Moench and seems to provide some evidence in support of the maintenance of this group of species as a valid genus".

The chromosome numbers so far known show that there are three chromosome series in the genus *Cassia*; one series with the basic number 6, another series with basic number 7 and the third with the basic number 8. In the light of this the splitting of the genus into two genera *Cassia* and *Chamæcrista*, the first genus including all species showing a polyploid series with basic numbers 6 and 7; and in the second genus all species with polyploid series with basic number 8, as suggested by Senn (1938 *b*), seems to be justifiable.

Summary

Meiosis in nine species of *Cassia* was studied. The chromosome numbers previously reported by the writer were confirmed in seven cases and in *C. obtusifolia* the previous report of $n = 13$ was corrected and here also the chromosome number $n = 14$ was recorded. The chromosome number for *C. alata* was recorded to be $n = 14$ and this number is in variance with the number $2n = 12$ reported by Senn (1938).

The number of bivalents in association with the nucleolus at diakinesis and the organization of the nucleolus at telophase I were studied. These observations are in conformity with Jacob's (1940) observations for other species of *Cassia*.

Secondary association was studied and a maximum association of 7 was noted and on this basis the basic number for the genus is inferred to be 7.

Polyploidy within the species was noted in two cases: *C. auriculata* and *C. alata*. In. *C. auriculata* 2 nucleate P.M.C.s were observed and these P.M.C.s divide regularly to give rise to 8 microspores, which are nonfunctional and degenerate.

The chromosome numbers with relation to growth habit was studied. No correlation between chromosome numbers and growth habit was found.

Taxonomically Senn's suggestion of splitting the genus into two genera *Cassia* and *Chamæcrista* on the basis of chromosome numbers was supported.

In conclusion I wish to express my sincere thanks to Professor A. C. Joshi and Professor J. Venkateswarlu for their kind advice and help.

The work, though started earlier, was completed with the help of the Government of India Scholarship in this University. In this connection I wish to thank the Government of India and the Andhra University authorities for their financial help and facilities respectively.

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INFLUENCE OF COMBINED NITROGEN ON FIXATION OF NITROGEN BY *AZOTOBACTER*

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Introduction

Horner and Allison (1944) reported that in the absence of free nitrogen *Azotobacter chroococcum* is able to utilise nitrogen from ammonia and from its easily decomposable compounds. The inhibitory effect of ammonia on nitrogen fixation was reported by Wilson (1952). Gainey (1936) observed that tolerance of nitrates by *Azotobacter* depended on strain characteristics. It was, however, not clear whether a strain accommodating itself to a given concentration of nitrates, would lose its power of fixing nitrogen because of the ready availability of nitrogen. During the decomposition of organic matter in soils, nitrogenous compounds formed may inhibit nitrogen fixation by *Azotobacter*, because of the availability of the nitrogen in them. The ability or otherwise of the organism to tolerate the presence of these substances is important, because of the probable effects of these substances on nitrogen fixation by *Azotobacter*. This paper reports on the effect of ammoniacal nitrogen, nitrate nitrogen, aspartic acid and peptone on nitrogen fixation by *Azotobacter* studied under laboratory conditions.

Materials and Methods

Fred and Waksman's medium (1928) was used for the growth of *Azotobacter*.

The substances studied and their concentration ranges in 100 c.c. of the medium were: (*a*) Ammonium sulphate from 0·1 to 10·0 mg. of NH_4–N, (*b*) Potassium nitrate from 0·1 to 3·0 mg. of NO_3–N, (*c*) aspartic acid from 0·1 to 10 mg. and (*d*) Peptone from 0·1 to 10 mg.

The effect of each substance at the different concentrations was studied in triplicates. The flasks were inoculated with one loopful of an active culture of *Azotobacter* isolated from Delhi soil of 48 hours' growth. The

characteristics of the strain have been mentioned elsewhere (Rao and Iswaran, 1959). The nitrogen contents of the culture liquids were determined, after 21 days of incubation at 32° C., by the Kjeldahl Gunning method.

Results

The effects of the different forms of nitrogen in different quantities are given in Table I in terms of mg. nitrogen fixed per gram of mannite at the end of the incubation period and after allowing for blanks.

Table I

Effect of different forms of nitrogen on nitrogen fixation by Azotobacter

Concentration in mg./100 c.c. of the medium	Nitrogen fixed (mg./g. of mannite)*			
	Ammonium sulphate	Potassium nitrate	Aspartic acid	Peptone
0·00	6·65	6·58	6·60	6·10
0·10	6·13	5·73	7·47	6·03
0·25	6·02	5·58	7·47	5·87
0·50	5·55	4·91	7·30	5·50
1·00	5·70	4·60	6·67	2·78
1·50	4·43	2·27	..	..
2·00	4·40	2·01	6·77	2·70
2·50	3·30	1·67	..	..
3·00	3·27	1·25	5·63	2·43
4·00	3·27	..	4·57	2·48
5·00	4·20	..	5·03	2·18
6·00	5·00	..	2·80	2·23
7·00	3·13	..	2·00	1·77
8·00	3·06	..	0·33	1·83
9·00	3·06	..	0·77	2·07
10·00	3·66	..	1·43	1·97

* Average of three determinations.

It could be seen from the data that the overall tendency of the organism was to decrease amounts of nitrogen fixation with higher ammoniacal nitrogen supplied. This relation is expressed by the equation

$$y = 4{\cdot}8384 - 1{\cdot}5920 \log_{10} x,$$

where y is the amount of nitrogen fixed per gram of mannite and x the amount of NH_4–N supplied in mg. per 100 c.c. of the medium.

It could also be observed that there was a progressive fall in nitrogen fixation by the organism with increase in concentration of NO_3–N in the medium. The nitrogen in the medium together with the nitrogen fixed, amounted to only 4·25 mg. when a concentration of 3·0 mg. of NO_3–N per 100 c.c. of the medium was used, while in a medium without any added nitrate nitrogen, the fixation of nitrogen by the organism amounted to 6·58 mg. This might be due to the azotase system in the cells of *Azotobacter* having been rendered ineffective wholly or partly by the nitrate ions.

The relationship between the amount of nitrogen fixed and the amount of NO_3–N supplied was significant and is expressed by the equation

$$y = 3{\cdot}7308 - 3{\cdot}3141 \log_{10} x$$

where y is the amount of nitrogen fixed per g. of mannite and x the amount of NO_3–N supplied in mg. per 100 c.c. of the medium.

Nitrogen fixation was stimulated when aspartic acid was added up to 0·5 mg. per g. of mannite, beyond that the fixation of nitrogen started decreasing.

The relation between the amounts of nitrogen fixed by *Azotobacter* and the concentration of nitrogen in the form of aspartic acid was significant and could be expressed in the form of the following equation

$$y = 5{\cdot}7482 - 3{\cdot}5405 \log_{10} x,$$

where y is the amount in mg. of nitrogen fixed per g. of mannite and x, the amount of nitrogen added in the form of aspartic acid.

A progressive decrease with fixation with increase of concentration of peptone in the medium was also observed. Similar decrease in nitrogen fixation was observed by Joshi (1934–35) when a strain of *Azotobacter* was cultured in peptone broth. This relationship could be best expressed by the equation

$$y = 3{\cdot}9008 - 2{\cdot}3344 \log_{10} x,$$

where y is the amount of nitrogen fixed in mg. per g. of mannite and x, the amount of peptone added in mg. per 100 c.c. of the medium.

DISCUSSION OF RESULTS

These results which accord with those of previous workers show that under ordinary soil conditions, the decomposition products of organic sub-

stances must be occurring either in amounts too small to affect the nitrogen fixing power of the organisms or they may be immediately acted on by ammonifying organisms to convert them into ammonia or ammoniacal compounds, which have been observed to have the least inhibitory or toxic action on the nitrogen fixation by *Azotobacter*. In the case of application of organic manures, considering the amounts applied, the actual amount of organic nitrogen may not be very high, because of the very high proportions of moisture and of insoluble minerals like sand, silt, etc., in the manures applied. So the decomposition products of organic matter may not be so high in concentration as to depress the fixation of nitrogen by *Azotobacter*. In the case of fertilizers, the concentration of the inorganic nitrogenous substances in soil solution may at times be high and cause serious inhibition of nitrogen fixation; however, the soil organisms other than *Azotobacter* may decrease the concentration by deriving their nutritionally required nitrogen from these compounds.

In the case of undisturbed virgin soils, the annual gain in soil nitrogen may be due to the unimpaired activity of the *Azotobacter*, where these external disturbing factors are absent. Under such conditions, when the stable equilibrium regarding soil organic matter and soil nitrogen is reached, the amounts corresponding to these, being dependent on the climatic conditions, the amounts of organic matter are hardly sufficient for giving rise to these toxic substances. If such conditions are created, *Azotobactor* in commonly observed forms ceases to exist or mutates to different forms which can thrive under these conditions when the nitrogen substances are less toxic to these mutants. One such organism was observed by Altson (1936) in Malayan soils rich in organic matter and nitrogen.

Summary and Conclusion

Different amounts of ammonium sulphate, potassium nitrate, aspartic acid and peptone supplying different amounts of nitrogen to the medium were added to the mannite solution. The materials were inoculated with a strain of *Azotobacter* isolated from Delhi soil and incubated at 32° C. for three weeks. Nitrogen contents of the materials were determined.

It was observed that the addition of nitrogen through these substances had an inhibitory effect on nitrogen fixations by *Azotobacter*. The relation between the amounts of nitrogn supplied and those fixed had been quantitatively worked out.

It could be observed from the quantitative expressions that ammoniacal nitrogen had the least and nitrate nitrogen the highest inhibitory effect on

nitrogen fixation by *Azotobacter*. In small amounts, nitrogen in the form of aspartic acid had a stimulatory effect on nitrogen fixation, but in higher doses it became even more toxic than potassium nitrate.

Peptone seemed to have a very strong inhibitory effect on nitrogen fixation by *Azotobacter*.

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THE SKULL OF *CROCODILUS PALUSTRIS* LESSON

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Introduction

In the structure of the palate, the auricular appendages covering the tympanic membrane, the snout, variations in size of the upper temporal fossæ in contrast with the orbits, fusion of the quadrate with the occipital constituents and the varying length of the symphysis, Crocodilia has progressed in evolution in relation to other reptiles. These characters seem to point to its Dinosaurian affinities but do not entirely support its descent from the latter. Despite the common interest in Crocodilia there is no authentic record of its anatomy in detail. Parker (1883) gave a general account of the structure and development of Crocodilia. Killian (1890) referred to the anatomy of the external ear appendages in Crocodilia. Shuno (1914) wrote an account of the chondrocranium of *Crocodilus*. Edgeworth (1935) described the cranial musculature of *Crocodilus* which was further enlarged by Kesteven (1942–45). Smith (1935) in the *Fauna of British India* has also given sketches of the skull of *Crocodilus palustris* and *Gavialis gangeticus*. However, the various bones in these diagrams are not labelled and the description is wanting in details. Recently Belliars and Schute (1955) have dealt with the external ear in *Alligator mississippiensis, Caiman latiorotris* and *Crocodilus niloticus*.

MATERIAL AND TECHNIQUE

The present observations were made on two well cleaned specimens of the skull and the lower jaw of *Crocodilus palustris* Lesson in the collection of the Zoology Museum of Government College, Ajmer. Most of the observations did not need any optical instruments, however, sutures between the various bones were ascertained by the help of a 'bull eye' lens.

OBSERVATIONS

General

The skull is roughly pyramidal in outline. The dorsal and the side bones are roughly pitted with numerous depressions and the composition of the palate is unique. The snout is nearly two to two and a half times as long as broad at the base. There is a strong ridge in front of the orbits. The interorbital ridge is feebly developed. The skull is monimostylic, akinetic and diapsidan.

The Cranium

The cranium or the brain case is divided into three regions: Occipital, Parietal and Frontal.

Occipital region

The posterior region of the skull or the occiput presents a ring-like form and is called the 'occipital ring'. The occipital constituents are distinct, the basioccipital forms the floor and the supraoccipital the roof of this region.

Basioccipital or the occipital basilare (Fig. 4) is situated between the lamellæ of the exoccipitals. The occipital condyle (Fig. 4) which hangs over it is solely contributed by the basioccipital, a view held by Kingsley (1925) with regards to the origin of the occipital condyle in contrast to the tripartite nature of the condyle propounded by Goodrich (1930). It may well be said that exoccipital lamellæ (Fig. 4) support it laterally but do not participate in the formation of the occipital condyle. A prominent projection of the basioccipital (Fig. 4) marks the basioccipital contribution in the formation of the occipital condyle.

The exoccipitals.—The exoccipitals are laterally disposed above the foramen magnum. The paroccipital process of exoccipital represents the fusion of opisthotic with the exoccipital. The outer end of the paroccipital process of exoccipital articulates with the quadrate, thereby resembling a condition found in Chelonia. Lying on either side of the supraoccipital is the wing-like expansion of the postfrontal arcade which is, in fact, partly fused with lateral expansion of the exoccipitals.

Supraoccipital.—The supraoccipital is reduced, it is separated posteriorly from exoccipitals by sutures and anteriorly it is covered and partly fused with the parietals (Fig. 4). In between the supraoccipital and wing-like expansion of lateral occipitals, lie on either side oblong air passages which put the two tympanic cavities in communication with one another (Fig. 4). The epiotic is fused with the supraoccipital. The exoccipitals meet above the foramen magnum, and as such, the supraoccipital does not border it dorsally. Through the foramen magnum are seen on either side small oval projections of the auditory bulla.

Parietal region

Lying in front at relatively higher level of the occipital region, is the parietal region. Its roof is formed by the united parietals, while the floor is contributed by the basisphenoid.

Parietals.—The fused parietals lie between the upper temporal fossæ which border them laterally (Fig. 1). The frontals anteriorly and the squamosal and the supraoccipital posteriorly bound the limit of the parietal. The skull is profusely pitted all over from the parietals to the snout. The basisphenoid or the sphenoideum basilare is a small projection extending from the pro-otic and is scarcely visible being covered by the massive pterygoid. The parasphenoids are absent. The preorbitosphenoid remains cartilaginous and represents low cartilaginous interorbital septum. The alisphenoid or pleurosphenoid or lateral sphenoid retains separate entity lying between the ventral surface of upper temporal fossa and basisphenoid.

Frontal region

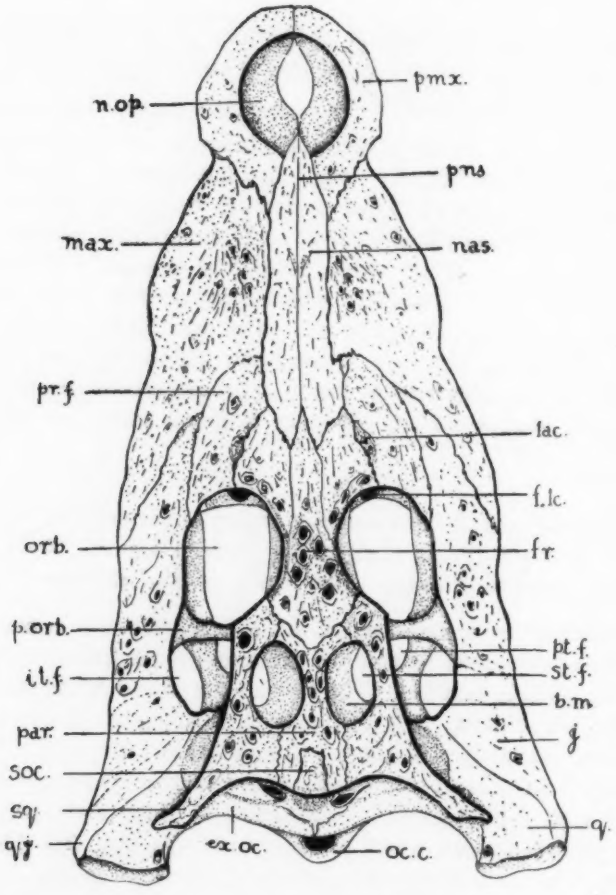
The frontal region is the anterior region of the cranium facing outwards from the axis and lies in front of the parietal region. The region chiefly comprises unpaired frontals. The region is also dorsally pitted.

Frontals.—The united frontals lie between the two orbits. The enormous size of the orbits reduces the extension of the frontal which is wedged by convex surfaces laterally. The frontals are paired in the young condition but with the ontogeny they fuse and form unpaired structure. The prefrontal lies anteriorly and the postfrontal posteriorly to the frontal. The prefrontal is in contact with nasal above and lacrymal laterally (Fig. 1). Dorsally each prefrontal has a characteristic ridge and ventrally it sends down a vertical process which meets the palatine (Fig. 2). The postfrontal with the squamosal forms a squamoso-postfrontal arcade separating the upper and the lower temporal fossæ (Fig. 3). The postfrontosquamosal

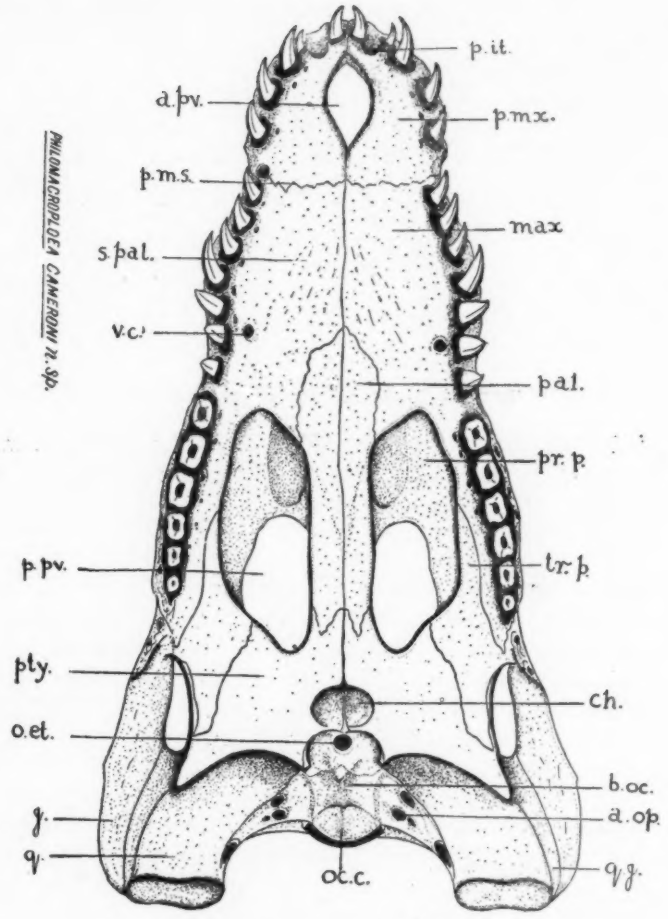
arcade partly fuses with the lateral occipitals and supports it. The post-fronto-squamosal arch also forms the supratemporal arcade bridging the infra- and supra-temporal fossæ (Fig. 3).

Olfactory capsule

The nasal capsule comprises the large nasals and prevomers (Fig. 1). A distinct bony septum nasi, a prolongation of the interorbital ridge, is also present.

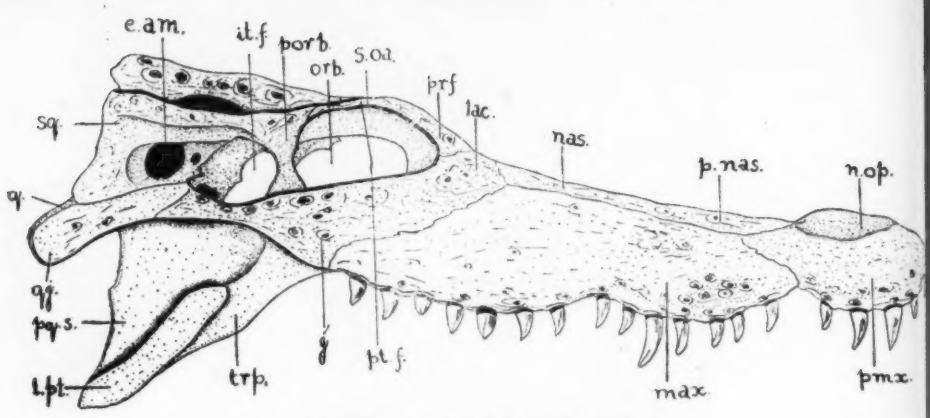


TEXT-FIG. 1. Dorsal view of the skull.



TEXT-FIG. 2. Ventral view of the skull.

Nasals.—The nasals are long and are extended up to the nasal opening. In *Gavialis*, nasals are far separated from the premaxillaries by maxillaries. In *Caiman*, nasals border the groove but do not form the nasal septum. In *Crocodilus palustris*, however, nasals reach the premaxillaries through the processus nasalis of premaxillæ (Fig. 1) which intervenes in the separation of nasals. The nostril is a solitary opening lying at the end of the snout surrounded by the premaxillaries on all sides (Fig. 1). The two long nasal canals running below the nasals are separated from one another by the down-



TEXT-FIG. 3. Lateral view of the skull.

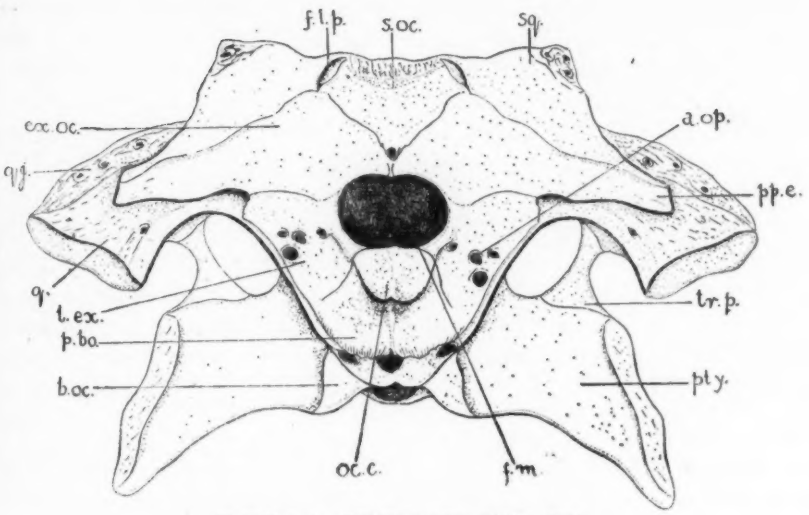
growths of the prevomers and pterygoids. The nasal canals extend from the anterior narial opening to the choanæ lying opposite the glottis (Fig. 2). The shifting of the choanæ far back behind the pterygoid is an aquatic adaptation in crocodiles.

Prevomers.—The term prevomer is generally applied, as true vomer is not present in fishes, amphibians and reptiles. The prevomers in *Crocodilus palustris* are oblong plate-like structures lying underneath the secondary palate and are invisible (visible in *Caiman niger*) as the ventral junction of the palatal constituents cover the prevomers entirely (Fig. 2). The internal nares are separated from one another by the opposed vertical plates of the prevomers.

The vomero-nasal openings or Jacobson's foramania are absent in *Crocodilus palustris* as also in other crocodiles and chelonia.

Auditory apparatus

In *Crocodilus palustris*, a curved slit-like opening running from just behind the orbit to the back of the skull represents external auditory meatus (Fig. 3). The tympanic cavity is surrounded by quadrate, quadratojugal, squamosal and the auricular plate. The tympanic cavity gives off numerous air passages into the surrounding bones. The air passage of the quadrate through a membranous siphonium communicates with Os articulare of the lower jaw (Fig. 6). The series of aerial openings existing on the posterior roof of the skull (Fig. 4) put both tympanic cavities in communication with one another. Attached to the tympanic membrane is a trifid cartilage with



TEXT-FIG. 4. Posterior view of the skull.

which articulates the bony columella auris, extending from the fenestra ovalis to the tympanic cavity.

Pro-otic

The pro-otic or otosphenoid remains a separate bone while the opisthotic fuses with the exoccipital and the epiotic with the supraoccipital respectively (Fig. 4). The pro-otic lies at a deeper level in the wide pterygo-quadrate space. The pro-otic is roughly triangular in outline. One of the arms, directed towards the basioccipital, is the anterior inferior process, and the other arm which is pointed towards the alisphenoid is the anterior superior process. The third arm is the parietal process pointing towards the exoccipital.

Upper Jaw

The upper jaw has paired premaxilla and maxilla. The premaxilla incorporates the anterior end of the snout while the maxilla bounds the skull laterally. A prominent jugal bone is present at the hinder margin of each maxilla (Fig. 3).

Premaxilla.—The premaxillæ are short and enclose a single nostril at the anterior margin of the snout. Dorsally at the anterior margin, paired openings represent the perforations caused by the lower incisor teeth (Fig. 1).

There are five unequal teeth in each premaxilla in comparison to four premaxillary teeth found in *Crocodilus siamensis*. The processus nasalis of premaxillæ intervenes in the separation of two nasals as it wedges between them (Fig. 1). The premaxillomaxillary suture (Fig. 2) is transverse in *Crocodilus palustris* in constrast to a 'W'-shaped suture recorded hitherto. In *Crocodilus siamensis* the premaxillomaxillary suture is present on the palate posteriorly.

Maxilla.—The elongation of the snout is due to the extension of the maxilla and not premaxilla. The maxillary teeth are 14 in number implanated in separate alveolar sockets (thecodont). Thus 16–19 teeth are present in the upper jaw out of which 5 belong to premaxilla. Both premaxilla and maxilla send palatal plates which contribute in the formation of the secondary palate (Fig. 2).

Jugal

Lying at the posterior margin of the maxilla dorsally over the ostransverum is a large jugal separated from the quadrate by an intervening bone, the quadratojugal (Fig. 3). The absence of a bridge between the jugal and squamosal enables the infratemporal vacuity to open freely behind.

Temporal space

Versluy's (1936) classification ascribes diapsidan variety to a Crocodilian skull as there are two temporal fossæ (supra and infra) and two temporal arcades (postfrontosquamosal and paraquadrate). The post-temporal fossa or foramen retrotemporalis of Siebenrock and its arcade are absent in *Crocodilus palustris*. The size of the upper temporal fossa bears an inverse ratio to that of lateral fossa.

Supratemporal fossa

The supratemporal fossa is reduced and is closed due to enormous development of dermal scutes (Fig. 1), which impart a superficial sculpturing to the skull. The superior temporal arcade is formed by the union of the postfrontal and squamosal (Fig. 3). The post-frontosquamosal arcade borders the outer limit of the upper temporal fossa and the inner boundary of the lower temporal fossa. The fused parietals separate the two upper temporal vacuities, while the squamosal bounds them posteriorly (Fig. 1). The ventral opening of supratemporal fossa is marked at the anterior surface of the otosphenoid and pleurosphenoid which are quite distinct components.

Infratemporal fossa

The infratemporal vacuity lies at a lower level than the upper vacuity. It occupies a side position and whence the name lateral temporal fossa.

This fossa usually opens behind, exposing part of the quadrate as the jugal fails to reach the squamosal (Fig. 3) anteriorly. The lateral fossa is separated from the orbit by a strong postorbital bar formed by a process of the postfrontal and jugal (Fig. 1). The quadratojugal, the jugal and the postfrontal form a strong superficial bridge with the squamosal from above. The infratemporal arcade is formed by the union of the jugal and the quadratojugal (paraquadrate).

Orbit

The orbits are large and reduce the size of the cranial cavity. The prefrontal, lacrymal (perforated), postfrontal, jugal and supraorbital arcade (Figs. 1 and 3) are the important members of the circumorbital series. The deeper margin of the orbit is partly separate from the infratemporal vacuity by a strong column formed by a downward process of the postfrontal uniting with the inner process of jugal and the ascending process of the transverum. It is better to apply the term postorbital to this buttress since it occupies the same position as the lateropostfrontal in other reptiles. The postorbital which is tripartite in origin imparts additional strength to the skull. The posterior palatine vacuities or the suborbital fossæ (Fig. 2) are very large and surrounded by ectopterygoids, palatines and maxillæ. The preorbital or the antorbital fossa between the lacrymal and maxilla reported to be present in other diapsidan skulls appears to have secondarily closed in crocodiles (Archæosaurs). The lacrymal is lying at the anterior margin of the orbit between the prefrontal and maxilla. The lacrymal has been recorded to be unperforated in crocodiles but in the present species, a distinct lacrymal foramen (Fig. 1) is present anterior to the orbit for the passage of the lacrymal duct (Fig. 1).

Bony palate

Crocodiles exhibit modification in the structure of the palate. The palatal components are paired palatines, pterygoids, transpalatines and prevomers. However, the prevomerine contribution in the formation of the palate is not visible externally.

Palatine.—The palatines are spatulate in form and meet one another along the middle line. Posteriorly, by a ' W '-shaped suture they are separated from the pterygoids. Laterally they are bounded by suborbital fossæ and anteriorly the maxillæ separate them by sutures. Dorsally each palatine meets a prolongation from prefrontal which partially splits the posterior palatine vacuity.

Pterygoid.—The pterygoids have undergone such a structural modification that internal narial opening is placed far back on the base of the skull. The choanæ is an oval depression bearing a pterygoidal suture dorsally and the basioccipital limits it ventrally. Each pterygoid is roughly triangular in shape. The lateral elevated margin of the pterygoid (Fig. 3) is marked over by a ectopterygoidal arm. Between the transpalatine and pterygoid a distinct suture persists. The sutural arrangements of the palate eliminates all possibilities of movements among its constituents and hence the skull is akinetic.

Transpalatine.—The transpalatine (ectopterygoid or transverum) (Fig. 2) is a triradiate bone lying between the maxilla, jugal, pterygoid and suborbital fossa. The transpalatine connects the pterygoid with maxilla and jugal. One of its arms descends down and contributes in the formation of the postorbital arch which consequently meets the ventral process of the jugal, and postfrontal and forms a strong column which effects the orbitotemporal partition. Dorsally a strong ectopterygoidal arm supports the maxilla and ventrally the third arm roofs over the lateral elevated part of the pterygoid.

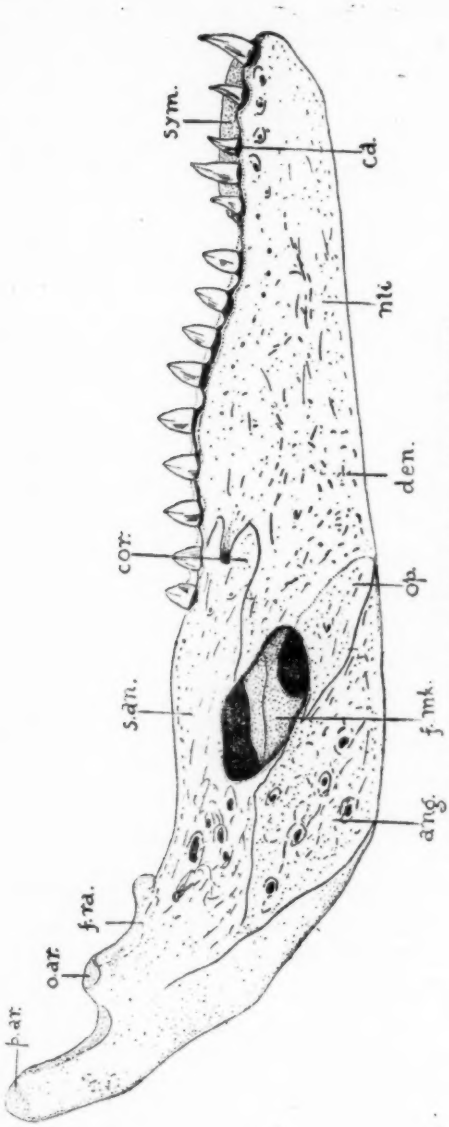
Suspensorium.—The quadrate is large and immovably fixed. It is directed backwards and is overlaid by the squamosal. The quadrate is surrounded by the paraoccipital process of exoccipital, quadratojugal and squamosal. Its external margin has an irregular double condylar facet for the articulation with the fovea articularis of the mandible. The quadrate movement is entirely cut off due to rigid articulation of the quadrate with the neighbouring bones.

Lower jaw

The mandibular rami form symphysis of variable length. Each ramus unites with its fellow of the other side anteriorly by a suture, and has six bones. The fossa meckelli and fovea articularis are also very distinct. The splenial enters the symphysis while the goniale is fused with the articular.

Articular

The articular (Fig. 5) lies at the anteroproximal part of the ramus mandibularis. Its upper, posterior and inner corners are perforated (Os articulæ) as recepticles for siphonium and, thereby, connecting the cavities of middle ear with the empty spaces enclosed within the mandible. The goniale or prearticular disappears or probably has fused with the articular. The postarticular or the processus retro-articularis lies behind the fovea articularis into which the condylar facet of the quadrate fits (Fig. 5).



TEXT-FIG. 5. The right half of the lower jaw, outer aspect.

Angular

The angular lies at the ventroproximal margin of the ramus between the articular and coronoid. The fossa meckelli lies at the mid-dorsal side of the angular (Fig. 5).

Suprangular

The supra or suprangular (Fig. 5) extends from the articular to the dentary. At the latter articulation it is bifid, and in this way forms the dorsal bone of the lower jaw. Ventrolaterally the fossa meckelli is an elliptical depression and a part of angular limits its boundary.

Coronoid

The coronoid is a small bone between dentary and angular and is partly hidden inside fossa meckelli (Fig. 5).

Splenial

The splenial in *Crocodilus palustris* is a small bone present at the inner aspect of the jaw. Its counterpart on the outer side is represented by an aperture (Fig. 5).

Dentary

The dentary contributes a major share in the formation of the distal half of the mandibular ramus. As in the upper jaw, the dentary bears thecodont teeth. The teeth are more or less conical and the sockets in which they fit lie over a ridge called ' crista dentalis' (Fig. 5). The dentary has 14 teeth overlapped laterally by those of the upper jaw. The 4th mandibular tooth is the strongest and the longest and fits into a lateral notch present at the premaxillomaxillary junction in the cranium.

SUMMARY

1. The skull is diapsidan, akinetic and monimostylic.

2. The supraoccipital is reduced and partly fused with the parietal, the exoccipital has lateral lamellæ and the basioccipital alone contributes in the condylar formation.

3. The parietals and the frontals are united. The prefrontal sends a downward process to the palatine, while the postfrontal forms the postfrontosquamosal arcade which partly fuses with the lateral occipitals.

4. The interorbital ridge is partly cartilaginous and partly membranous. The former is represented by the preorbitosphenoids. The basisphenoid is a small protuberance facing the pro-otic,

5. The nasals reach the premaxilla which surrounds the solitary nostril. The nasal channels run below the pseudopalate. The septum nasi is the anterior prolongation of the interorbital septum.

6. The tympanic cavity perforates the neighbouring bones with air passages, which are thus pneumatic in character. The auditory bulla is visible through foramen magnum.

7. The elongation of the snout is due to maxilla while the premaxilla has processus nasalis to separate the nasals. The premaxillomaxillary suture is transverse. The upper jaw has 19 thecodont teeth of which 5 are on the premaxilla and 14 on the maxilla.

8. The postorbital is a contribution of jugal, transpalatine and postfrontal. It divides the orbitotemporal vacuity in two parts, the orbit and the infratemporal fossa.

9. The supratemporal fossa is small and is closed. The lateral temporal vacuity is open behind. The paraquadrate is a bridge formed of the quadrate and the quadratojugal.

10. The lacrymal is clearly perforated while the supraorbital arcade is formed by the union of the pre- and the postfrontals.

11. The pseudopalate is formed by the palatal projection of the palatal constituents. The structural modification of the pterygoids brings the choanæ farther back at the base of the skull. The transpalatine is triradiate, the palatine is spatulate and the pterygoid is triangular.

12. In the lower jaw there are 14 thecodont teeth. The splenial enters the symphysis. The elevated lateral expansion of pterygoid articulates with the retroarticularis process.

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ABBREVIATIONS

ang. .. angular

a.op. .. air opening

a.pv. .. anterior palatine vacuity

bm. .. scute-like bony matter

b.oc. .. basioccipital

cd. .. crista dentalis

ch. .. choanæ

cor. .. coronoid

den. .. dentary

e.a.m. .. external auditory meatus

ex.oc. .. exoccipital

f.lc. .. foramen lacrymale

f.l.p. .. foramen lacrum posterius

f.m. .. foramen magnum

f.mk. .. fossa meckelli

fr. .. frontal

f.ra. .. fovea retroarticularis
i.t.f. .. infratemporal fossa
j. .. jugal
lac. .. lacrymal
l.ex. .. lamellæ of exoccipital
l.pt. .. lateral elevation of pterygoid
nas. .. nasal
n.li. .. nervous alveolaris
n.op. .. nasal opening
max. .. maxilla
o.ar. .. osarticularis
oc.c. .. occipital condyle
o.et. .. median opening of the eustachian tube
op. .. operculæ (counterpart of splenial)
orb. .. orbit
pal. .. palatine
par. .. postarticular
p.b.o. .. projection of basioccipital
p.it. .. perforation of incisor tooth
p.m.s. .. premaxillomaxillary suture
pmx. .. premaxillæ
p.nas. .. processus nasalis
p.ns. .. processus nasalis of premaxilla
pp.e. .. paraoccipital process of occipital
p.orb. .. postorbital
p.pv. .. posterior palatine vacuity
pr.p. .. prefrontal process
pr.f. .. prefrontal
pt.f. .. postfrontal
pty. .. pterygoid
pq.s. .. pterygoid quadrate space
q. .. quadrate
qj. .. quadratojugal
s.an. .. supra-angular
s.oc. .. supraoccipital
s.oa. .. supraorbital arch
s.pal. .. secondary palate
s.t.f. .. supratemporal fossa
sym. .. symphysis
sq. .. squamosal
trp. .. transpalatine
vc. .. vascular channels opening into alveoiar sinus

SOURCE OF ACETYLCHOLINE IN THE FROG'S HEART ON STIMULATION OF THE VAGUS NERVE

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The heart is known to contain endogenous acetylcholine (Burn, 1956). If the vagus nerve to the stomach of frog or dog is stimulated, the liberated acetylcholine appears to come not from the nerve but from the stomach muscle itself (Singh, Sharma and Bhatnagar, 1959 *b*). Loewi (1921, 1922) has shown that when the vagus nerve to the frog's heart is stimulated, acetylcholine is liberated, and it has been presumed that it comes from the vagal nerve endings. But if the heart contains endogenous acetylcholine, then there is the possibility that the acetylcholine liberated on stimulation of the vagus nerve to the heart might originate at least partly, if not wholly, from the heart muscle and not from the nerve endings. It was reported in a previous paper (Singh, Sharma and Bhatnagar, 1959 *a*, *b*), that sometimes stomach muscle of the frog does not release any acetylcholine on stimulation of the vagus nerve, but does so on direct electrical stimulation. In the present research, similar instances were found with the frog's heart. Acetylcholine was not released or released in small quantities on stimulation of the vagus nerves; but it was released on direct electrical stimulation if there was no release on stimulation of the vagus nerves, or in much greater quantity than that liberated on nervous stimulation.

Experimental

These experiments were performed on the heart of the frog, *Rana tigrina*. The heart was dissected out with both the vagus nerves attached. It was washed free of blood by repeated washing with eserinised Ringer's solution for half an hour. It was then placed in a tilted petri-dish containing 15 c.c. of eserinised Ringer's solution for one hour; it usually beats spontaneously. This was done for one hour to note any liberation of acetylcholine by the spontaneously beating heart; the heart beats from about

2 to 3 hours. The Ringer's solution was then replaced afresh and both the vagus nerves were placed on a pair of electrodes and stimulated maximally with induction shocks for one hour. At the end of the period of stimulation, the Ringer's solution was collected and tested for acetylcholine. The heart stops on stimulation of the vagus nerves, and does not resume beating on cessation of stimulation, but responds to direct electrical stimulation.

The nerves were then cut out and the heart placed in a small muscle chamber containing 15 c.c. of eserinised Ringer's solution and stimulated with induction shocks twice every 15 seconds for one hour. The Ringer's solution was then tested for acetylcholine.

The acetylcholine was assayed on sensitive leech preparations, which responded to 1 in 1,000 million acetylcholine. These experiments were performed at room temperature, 22–24° C. The composition of the Ringer's solution was as described previously (Singh, Sharma and Bhatnagar, 1959 *a*, *b*, *c*).

Results

The spontaneously beating heart.—In 26 experiments the spontaneously beating heart of the frog showed no release of acetylcholine (Table I). The frog's heart therefore does not require any release of acetylcholine for its rhythmic activity.

Effect of vagus stimulation.—Both the vagus nerves were stimulated in 26 experiments. In 11 experiments, stimulation of vagus nerves did not show any detectable release of acetylcholine; in 15 experiments, there was release (Table I). The period of maximal stimulation, that is, one hour, is sufficient to fatigue the nerves.

Effect of direct stimulation.—Direct electrical stimulation caused release of acetylcholine in all the 26 experiments (Table I). The amount released was very variable. But as with stomach muscle (Singh, Sharma and Bhatnagar, 1959 *b*), direct electrical stimulation is more potent than nervous stimulation for release of acetylcholine.

Discussion

There is no doubt that acetylcholine may be liberated on stimulation of the vagus nerve to the frog's heart, but there is also the curious fact that the heart may stop on stimulation of the vagus and yet there may be no detectable release of acetylcholine. It may be presumed that the acetylcholine is liberated in very small amounts, so that it is all absorbed by the

TABLE I

Acetylcholine released from frog's heart as a result of vagus and electrical stimulation

Number of experiment	Acetylcholine in nanograms released from 1 gm. of tissue per hour			
	Spontaneously beating heart	Vagus stimulation	Electrical stimulation	Increase %
1	0	0	46	..
2	0	0	14	..
3	0	0	62	..
4	0	0	375	..
5	0	51	166	225
6	0	0	400	..
7	0	26	80	200
8	0	40	120	200
9	0	10	100	900
10	0	75	375	400
11	0	36	180	400
12	0	50	350	600
13	0	0	150	..
14	0	0	230	..
15	0	11	34	209
16	0	8	24	200
17	0	0	40	..
18	0	0	241	..
19	0	0	82	..
20	0	0	23	..
21	0	26	130	400
22	0	34	100	200
23	0	120	300	150
24	0	40	120	150
25	0	60	300	400
26	0	65	300	360

receptors, or the release of acetylcholine is intracellular in amounts too small for external leakage.

The fact that acetylcholine can be liberated by direct electrical stimulation after the vagus nerves have been fatigued, or if they do not release acetylcholine on stimulation, suggest that the substance has originated from the muscle cells and not the nerve endings. The spontaneously beating heart does not release acetylcholine, so that the release on direct electrical stimulation suggests some specific effect of electric current, a phenomenon of importance in release of acetylcholine by the action potential of nerve.

Electrical stimulation of the brain is known to release acetylcholine. If electrical stimulation has a specific effect in releasing acetylcholine, then electrical transmission in the brain and other parts of the nervous system is a possibility. This view is supported by the fact that the cerebellum, which is so richly endowed with synapses, is poor in acetylcholine (Quastel, 1955).

Summary

1. The vagus nerves of the frog's heart were stimulated in 26 experiments. In 11 experiments, there was no detectable release of acetylcholine; in 15 there was release in variable amounts.

2. Direct electrical stimulation caused release of acetylcholine in all the 26 experiments, in amounts greater than that released by nervous stimulation after the fatigue of the vagus nerves, and also in those experiments in which the stimulation of the vagus nerves did not show any detectable release of acetylcholine. It is concluded, therefore, that the acetylcholine liberated on stimulation of the vagus nerves comes from the heart muscle cells and not the nerve endings.

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INDUCTION OF POLYPLOIDY IN WATERMELON

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Introduction

The development of seedless watermelon (*Citrullus vulgaris* Sehrad) in Japan by crossing induced tetraploids with the parent diploids is a good example of the successful exploitation of the technique of chromosome doubling through colchicine application in modern plant breeding. Watermelon is cultivated widely in India and its fruits are very popular particularly during summer. Studies on the production of triploid seedless watermelon were commenced at the Indian Agricultural Research Institute in 1956 and the results of this study are summarised in this report.

Material and Methods

Seeds of the watermelon varieties Asahi Yamato and Farrukhabadi maintained by the Plant Introduction Section of the Botany Division of the Indian Agricultural Research Institute were sown in the field for treatment with colchicine. The methods of colchicine application were:—

(*a*) Germinating seeds in 0·1, 0·2, 0·3 and 0·4% colchicine solution.

(*b*) Treatment of the growing point in the cotyledonary stage with colchicine applied either as an aqueous solution or in the following three media.

(1) Colchicine in glycerine (0·2 and 0·4% colchicine in 10% glycerine).

(2) Colchicine emulsion suggested by Warmke and Blakeslee (1939) for induction of polyploidy in *Nicotiana*. The colchicine concentrations used by us were 0·2 and 0·4%.

(3) Colchicine in agar (1% colchicine and 2% agar, mixed in equal parts).

Experimental Results

Effect of treatments.—The treatment of sprouted seeds proved to be too drastic at all the concentrations tried. The percentage of mixoploids and tetraploids obtained from various treatments of the growing point is given

in Table I. It would be observed from the data that the maximum frequency of polyploids (total and mixoploids) in both Asahi Yamato and Farrukhabadi, were obtained by treating growing points with 0·2% colchicine emulsion. The next best treatment was the application of 0·4% colchicine solution, as drops, to the growing point for one hour daily on four consecutive days.

TABLE I

Results of different colchicine treatments

Variety	Concentration (%) and medium of application	Method of treatment	No. of plants treated	Tetraploid or mixoploid	% Success
Farrukhabadi	0·4 col. solution	Drop	39	14	35·9
	0·2 col. solution	Drop	45	4	8·66
	0·4 col. emulsion	Drop	36	All died	..
	0·2 col. emulsion	Drop	48	19	39·59
	0·5 col. agar	Smeared on growing point	46	1	2·17
Asahi Yamato	0·4 col. solution	Drop	62	10	16·1
	0·2 col. solution	Drop	41	3	7·3
	0·4 col. emulsion	Drop	38	All died	..
	0·2 col. emulsion	Drop	43	7	16·29
	0·5 col. agar	Smeared on growing point	29	1	3·45

Morphological Characteristics

The tetraploid plants exhibited the usual gigas features characteristic of autopolyploids. Thus these plants, in general, showed greater growth and vigour as compared to the diploids (Figs. 1 and 2). They had thicker, more prominently ribbed stems with shorter internodes; larger, thicker and more deeply lobed leaves of a dark-green colour having a lower length/breadth index of the terminal lobe and larger anther lobes in the male and larger ovaries in the female flowers. The flowering in tetraploid Farrukhabadi was delayed by 10–15 days. The seeds in tetraploid fruits were larger but fewer, more round and characterized by fissures and wrinkles on the seed-coat (Figs. 3, 4 and 5). Tetraploids of Asahi Yamato were very late and

since they became heavily infested with beetles and damaged by rains no fruit set could be obtained. Consequently seed setting studies had to be confined only to Farrukhabadi.

Meiotic behaviour

Meiosis was studied during microsporogenesis in two tetraploid plants. In diploids, 11 bivalents were invariably seen at first metaphase. Ring bivalents with chiasmata in both the arms of the chromosomes were most common (Fig. 6). In the tetraploids various combinations of univalents, bivalents, trivalents and quadrivalents, making up a total of 44 were observed. The results of pairing analysis and multivalent frequency at metaphase I and the types of quadrivalents that occurred are given in Tables II, III and IV.

TABLE II

Pairing frequency in diploid and tetraploid watermelon at metaphase I

Plant No.	Chromosome No.	No. of PMC's studied	Mean frequency per nucleus of			
			Univalents	Bivalents	Trivalents	Quadrivalents
1	22	20	..	11	..	..
2	44	31	0·225	9·1	0·225	6·23
3	44	18	0·11	10·78	0·11	5·5

TABLE III

Frequency of multivalents in tetraploid watermelon at metaphase I

Plant No.	No. of cells with quadrivalent + trivalent frequency of												Multivalents	
	0	1	2	3	4	5	6	7	8	9	10	11	Mean/ cell	Coefficient of realization
1	0	0	0	1	5	4	5	6	8	0	1	1	6·45	0·586
2	0	0	0	2	2	4	5	3	2	0	0	0	5·61	0·51

It will be observed from Table III that 11 quadrivalents, which is the maximum number possible, was observed in one cell (Fig. 7). The coeffici-

ent of realization of multivalents ranged from 0·51–0·56. Among the quadrivalents the symmetrical types were the most prevalent. The mean chiasma frequencies per nucleus and per bivalent are given in Table V. It is interesting that in spite of the small size of the chromosomes and the low frequency of chiasmata, the coefficient of realization of multivalents is fairly high.

TABLE IV

Frequency of different types of quadrivalents in tetraploid watermelon at metaphase I

Plant No.	No. of quadrivalents of type				Total No. of quadrivalents studied	Quadrivalents with symmetrical configuration (%)
	3 X ta		4 X ta			
	X	Y	⟊	[ ]		
1	10	10	14	159	193	89·1
2	1	5	4	89	99	92·8

TABLE V

Chiasma frequency in diploid and tetraploid watermelon at metaphase I

Material	No. of PMC's studied	No. of config. with X ta					Chiasmata		
		0	1	2	3	4	Total	Per cell	Per bivalent
Diploid ..	20	..	..	220	..	..	440	22·00	2·0
Tetraploid	49	9	49	436	44	284	2045	41·73	1·8

In diploids the anaphase disjunction was regular. In the tetraploids, there were a few lagging univalents in some cells. At metaphase II, 22 chromosomes were most commonly seen at each pole (Fig. 8). In a few cells, however, there was an unequal number of chromosomes at the two poles probably due to the non-inclusion of lagging chromosomes into either of the groups. Regular equational division of the chromosomes took place at second anaphase. Tetrads were formed after the completion of the second division and were of the tetrahedral type.

Fruit and seed setting

Data on seed setting in diploid and tetraploid fruits developed under open pollinated condition and in selfed tetraploid fruits are given in Tables VI and VII. As indicated by the data, the mean seed set per fruit in the tetraploid following selfing is considerably lower than the seed set obtained in open pollinated tetraploid and diploid fruits. Further, the percentage of shrivelled seeds in tetraploid is 17·73% higher than that of the diploid. Only one fruit could be obtained from 72 selfed tetraploid flowers. In other cases, the fruits developed normally for about a week, but later started shrivelling and finally dropped off.

TABLE VI

Seed setting in diploid and tetraploid watermelon

Material	Total No. of seeds per fruit	No. of shrivelled seeds	% shrivelled seeds
Diploid ..	681	157	23·06
Tetraploid ..	446	182	40·79

TABLE VII

Seed setting in tetraploids

Pollination	No. of fruits obtained	Mean No. of seeds per fruit
Open pollinated ..	4	526
Selfed ..	1	364

Sucrose content of 2x and 4x fruits

The sugar content, determined by a hand refractometer, was 8% in the fruits of both diploid and tetraploid Farrukhabadi. Chromosome doubling therefore did not result in any alteration in this character.

Triploid seeds

One fruit obtained from crossing tetraploid and diploid Farrukhabadi yielded 67 seeds. The seeds on dissection showed a very poor embryo development and they germinated only when the seed coats were removed.

Triploid plant

Three triploid plants of the variety Farrukhabadi were raised during 1958. The seedlings were abnormal and poor in growth. There were several rudimentary seeds in a fruit set in one plant. These seeds were soft and the fruit could be eaten without removing the seeds. However the formation of a large number of ex-embryonate seeds may detract the value of a triploid Farrukhabadi fruit. Owing to unfavourable weather conditions and heavy infestation with pumpkin beetle, the plants were unhealthy and fruit setting very poor.

DISCUSSION

Success in the commercial production of triploid watermelons would depend upon (*a*) the availability of a suitable method of chromosome doubling, (*b*) the ease with which crosses between tetraploids and diploids can be made, (*c*) germinability of $3x$ seeds, (*d*) shape, size and yield of fruits in the triploid, and (*e*) the absence of seeds in the fruits set on the triploid plant. The results obtained by the Japanese workers (Kihara, 1951) and during the present study indicate that chromosome doubling can be induced in watermelon without much difficulty by applying colchicine on young seedlings, either as an emulsion comprising colchicine (0·2%), stearic acid, morpholine and lanoline or as drops (0·4%). The tetraploids breed true for chromosome number and there is hence no difficulty in maintaining them. Crosses between $4x$ and $2x$ plants seem to yield variable results and seed setting in such crosses is apparently conditioned by environmental conditions (Kihara, 1951). While we could not get any fruit setting in $2x \times 4x$ crosses one fruit was obtained in crosses between $4x \times 2x$ plants (the number of flowers pollinated was 29). Kihara (1951) found that in three sets of crosses made between June 14 and July 15, July 16 and August 4 and August 5 onwards, the percentage of fruit setting was 92·3, 25·0 and 10·7 respectively. It should therefore be possible to obtain a high percentage of success in $4x \times 2x$ crosses by fixing the optimum period for hybridization work.

The production of triploid seeds through artificially pollinating tetraploids with diploids will involve considerable labour and expense. Stebbins (1956) has expressed the view that the large amount of labour involved in this

work militates against the success of these triploids in the United States. By growing diploids and tetraploids near each other and by using suitable marker genes, the need to carry out artificial hand-pollination can be obviated. A single gene controlling dark parallel striping on the fruit serves as a suitable genetic marker. If this dominant gene is present in the diploid pollinator, fruits of triploids will be striped while tetraploids will not show striping; thus the two types of plants derived from a tetraploid can be readily distinguished.

Since the triploid seeds needed for raising seedless watermelons are obtained from $4x \times 2x$ crosses, $4x$ seeds resulting from self-pollination tend to get mixed up with $3x$ seeds. Yamashita *et al.* (1957) have found $2x$ seeds also in the commercial $3x$-seed samples. It is thus necessary to have means for separating the $4x$, $3x$ and $2x$ seeds in a mixture. Fortunately, this can be easily accomplished since $3x$ seeds occupy an intermediate position between those of $2x$ and $4x$ in the length, width, thickness and weight of seeds (Yamashita *et al.*, 1957; Shimotsuma and Matsumoto, 1957). The $3x$ seeds, however, show a poor germination. The testa of the $3x$ seeds is thick and rugged and in many seeds the embryos are shrivelled. A probable reason for these abnormalities is the deviation from the normal 2:3:2 chromosome number relationship among the embryo, endosperm and maternal tissue. Thus, the chromosome numbers in these three organs in a $3x$ seed would be 33, 55 and 44 respectively as against 44, 66 and 44 in the tetraploid or 22, 33 and 22 in the diploids. Such a deviation from the chromosome number proportions in these tissues may lead to various types of somatoplastic sterility (Brink and Cooper, 1947). We found that the germination of the $3x$ seeds is vastly improved when the seedcoat is partially removed. Also, Yamashita *et al.* (1957) observed that complete germination can be obtained by a careful selection of healthy seeds and by maintaining the temperature of the hot-bed in which the seeds are sown at 30° C.

The yield of fruits in the triploid plants is directly related to the extent of stimulation provided by pollination. Diploid plants are therefore sown near triploids to act as pollen donors. Tetraploids can also be grown for this purpose but diploids are preferable in view of the high degree of pollen sterility found in the tetraploids. Colocynth is grown in Japan as pollinator with satisfactory results (Yamashita *et al.*, 1957).

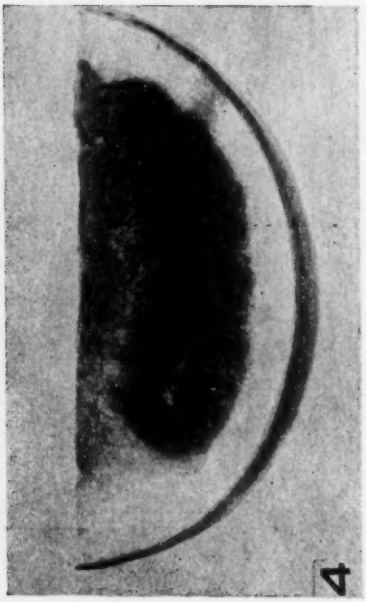
A major problem in the production of seedless watermelons is the development of the ovule in some triploid combinations to a hard seedcoat, without embryo. These hardened seeds in the botanically seedless fruits are undesirable from the consumer's point of view, although such seeds are

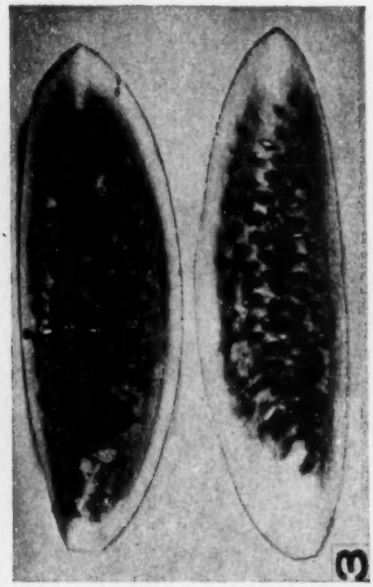
sometimes soft and edible. The Farrukhabadi triploid produced by us had a large number of such seeds. Yamashita *et al.* (1957) have found that there are varietal differences with regard to the tendency to form seeds without embryos. While in many Japanese varieties of watermelon the ovules showed no development in the triploid, some varieties from the United States, Spain and Pakistan gave unsatisfactory results. Thus, it is very important that the correct parents are chosen for the production of seedless fruits. Though it is difficult to draw any generalised inference from the behaviour of the single Indian variety used in the present study, it appears that it may be preferable to use Japanese varieties in such work since the experience of the Japanese workers with varieties from the Indian sub-continent has also been unfavourable and since it has been found by Singh and Joshi (1955) that some Japanese varieties of watermelon are outstanding in their performance when grown in parts of India.

The fruits of triploid watermelon may offer a suitable material for studying the biochemical effects of seedlessness. We could estimate only the sucrose content in $2x$, $3x$ and $4x$ fruits and no significant difference was found. However, quantitative and even occasionally qualitative alterations in biochemical characters following chromosome doubling are not uncommon both in induced (Eigsti and Dustin, 1955) and naturally occurring polyploids (Janaki Ammal and Sundara Raghavan, 1958) and it would be extremely interesting to study the triploid fruits from this view-point since, in addition to changes resulting from the increased chromosome number, there may also be changes arising from seedlessness in such fruits.

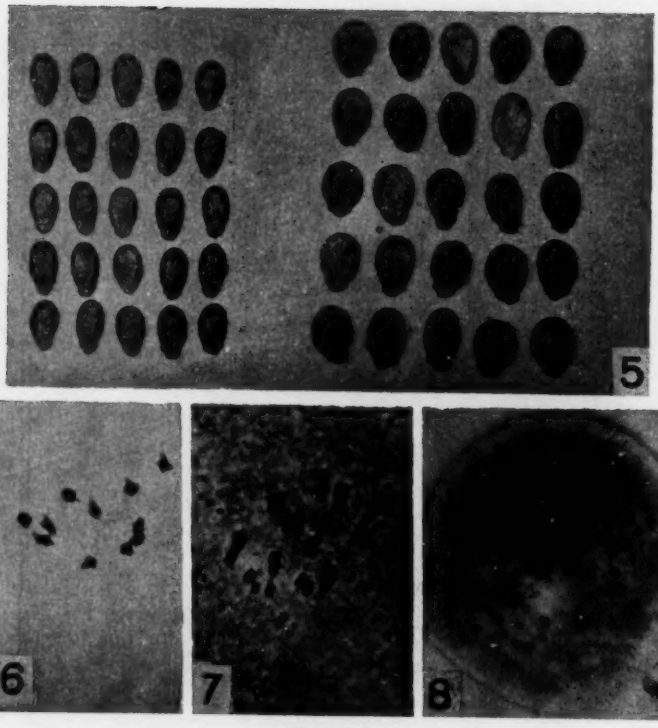
SUMMARY

Several colchicine treatment methods were tried in the varieties Farrukhabadi and Asahi Yamato of watermelon in order to induce chromosome doubling in them. Treatment of seedlings by drop method in the cotyledonary stage with an emulsion comprising 0·2% colchicine, stearic acid, morpholine and lanoline gave the highest percentage of affected plants (38·59%). Next in efficiency was the periodic application of drops of 0·4% colchicine on the growing point. The tetraploids had all the usual characteristics associated with autopolyploidy and during meiosis in a microsporocyte 11 quadrivalents were observed. The mean frequency of quadrivalents per cell was 5·91. Seed fertility in tetraploid watermelon was much reduced and from $4x \times 2x$ crosses in Farrukhabadi one fruit containing 67 seeds was obtained. The $3x$ seeds germinated only when the seed coat was partly removed and the fruit set on a $3x$ plant had seeds without embryo. There appears to exist genetic variability with regard to the ability of a strain to





FIGS. 1–4



FIGS. 5–8

yield a completely seedless triploid and it is consequently necessary to produce triploids of many varieties and select the desirable ones for the commercial production of seedless watermelon.

ACKNOWLEDGEMENTS

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EXPLANATION OF PLATES

PLATE I

FIG. 1. A diploid plant with fruits of the variety Farrukhabadi.

FIG. 2. A tetraploid plant of the variety Farrukhabadi. The tetraploid is late and forms fruits about a fortnight after fruit setting has commenced in the diploid.

FIG. 3. Cut portions of fruits of diploid (below) and tetraploid (above) Farrukhabadi showing the relative frequency and size of seeds.

FIG. 4. Cut portion of a fruit from the cross tetraploid × diploid, showing the very low seed setting.

PLATE II

FIG. 5. Seeds of diploid (left) and tetraploid (right) Farrukhabadi.

FIG. 6. Metaphase I in diploid watermelon showing 11 bivalents.

FIG. 7. Metaphase I in the tetraploid showing 11 quadrivalents.

FIG. 8. Metaphase II in the tetraploid with 22 chromosomes at each pole.
Magnification: Fig. 6, ×1,700. Figs. 7 and 8, ×1,080.

THE YEAST NUCLEUS

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Introduction

The discovery of the nucleus in a living yeast cell (Nägeli, 1844) closely followed that of Brown (1831; Sharp, 1934) in plant cells. But yet, while the progress in our knowledge of the structure and behaviour of the nuclei of higher organisms has been orderly and phenomenal, the original identification of the nucleus of the yeast cell itself has been questioned and a variety of cell organelles have been suggested to represent its nuclear 'apparatus'. This is well illustrated by the mutually contradictory conclusions drawn from recent publications in the two latest reviews on the subject (Nickerson, 1957; Winge and Roberts, 1958).

While the nucleus is visible in many types of living cells of higher organisms, it is generally invisible in living yeast cells. The confused state of our knowledge of its structure is only a corollary to the lack of availability of the living nucleus for comparison and reference. Henneguy's (1896) casual record of having seen a nucleus with a nuclear membrane and a nucleolus, in living and stained preparations of a red yeast, lacked confirmation, since later investigators observed it only as a homogeneous body in living cells. Whether the physiological age of the cells at the time of examination had any relation to this divergence of opinion remained long unexplored.

Disagreements Regarding Identification

When the organelle appears homogeneous in the living condition (Wager, 1898; Wager and Peniston, 1910; Sinoto and Yuasa, 1941; Lindegren and Rafalko, 1950; Mundkur, 1954) and is seen after staining as a dense structure (Wager, 1898; Wager and Peniston, 1910; Mundkur, 1954) or as a vesicle delimited by a membrane and containing formed structures inside (Sinoto and Yuasa, 1941) judgment as to which one of them is the true picture of the nucleus becomes questionable. The nuclear membrane and structures inside are likely to be dismissed as artefacts (Mundkur, 1954) if structural differentiation was not visible in the living condition.

If the organelle appears homogeneous in the living as well as stained preparations, its nuclear nature becomes suspect and is liable to be identified as a 'nucleolus' (Wager and Peniston, 1910) or as a 'centrosome' (Lindegren, 1949). If there are areas staining differentially in that structure they are likely to be considered as lying on the surface of that organelle (Wager and Peniston, 1910; Lindegren, Williams and McClary, 1956).

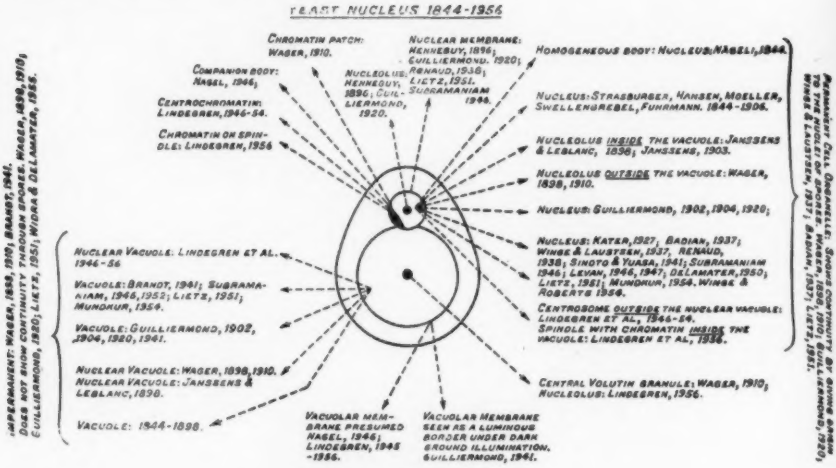
It is in such a context that the vacuole, visible in living cells, began to loom large in discussions on the yeast nucleus. The records of the presence of a network (Wager and Peniston, 1910) or of bodies extending into it (Lindegren, Williams and McClary, 1956), in stained preparations, are at variance with the affinity of the living vacuole for neutral red (Guilliermond, 1941) like the vacuoles of plant cells. The vacuole itself does not appear to be a permanent organelle of the yeast cell (Guilliermond, 1941; Caspersson and Brandt, 1941; Subramaniam, 1946; Aswathanarayana, 1956 *b*, 1958; Thyagarajan, 1956).

The Limitations of Fixation and Staining Procedures

The problem becomes more difficult when, as in many yeasts, the nucleus is invisible in the living condition at the time the investigations are carried out. The identification then of a particular organelle as the nucleus has necessarily to be based on studies of stained preparations under an ordinary microscope or of ultra-thin sections under an electron microscope (Agar and Douglas, 1957; Hashimoto, Conti and Naylor, 1958). The first step in such procedures is the proper fixation of the cells. When there is a doubt as to which organelle is the nucleus, the choice of a fixative becomes arbitrary. When the living nucleus is not available as a 'standard', an evaluation of its reaction to fixatives with a view to choose those which would give a life-like preservation becomes impossible.

This difficulty becomes magnified when it comes to the choice of suitable staining procedures. Hæmatoxylin, the stain of choice of earlier investigators, is a regressive stain. The stage to which the cells have to be destained to reveal the correct picture of the nucleus becomes purely arbitrary and empirical in the absence of the living nucleus for comparison and reference. "The Feulgen technique can lay claim to marked specificity in dye binding but has an analogous weak point in its initial acid hydrolysis step which is likewise of arbitrary duration and intensity" (Nickerson, 1957, p. 34). The Feulgen technique reveals only the location of the DNA in the cell. To determine whether the DNA is uniformly dispersed in the nuclear matrix (Mundkur, 1954) or whether it occupies only a limited area (Lietz, 1951) the living nucleus should be available for comparison. When there is no

agreement as to which organelle of the yeast cell is its nucleus or what its real structure is, it would be futile to discuss supernumerary mitoses and binucleate stages (Winge and Roberts, 1954 *a*, *b*; Roberts and van der Walt, 1959). In the context of the limitations of classical staining procedures, the views of various investigators from 1844–1956 given in ***Pictographic Summary I*** have only a historical interest.


YEAST NUCLEUS 1844-1956
CHROMATIN PATCH: WAGER, 1910.
NUCLEAR MEMBRANE: HENNEBUY, 1896; GUILLIERMOND, 1920;
COMPANION BODY: NAGEL, 1946;
NUCLEOLUS: HENNEBUY, 1896; GUILLIERMOND, 1920.
RENAUD, 1938; LIETZ, 1951. SUBRAMANIAM 1946.
HOMOGENEOUS BODY: NUCLEUS: NÄGELI, 1844.
NUCLEUS: STRASBURGER, HANSEN, MOELLER, SWELLENGREBEL, FUHRMANN. 1844-1906.
CENTROCHROMATIN: LINDEGREN, 1946-54.
CHROMATIN ON SPINDLE: LINDEGREN, 1956
NUCLEOLUS INSIDE THE VACUOLE: JANSSENS & LEBLANC, 1898; JANSSENS, 1903.
NUCLEOLUS OUTSIDE THE VACUOLE: WAGER, 1898, 1910.
NUCLEUS: GUILLIERMOND, 1902, 1904, 1920;
NUCLEUS: KATER, 1927; BADIAN, 1937; WINGE & LAUSTSEN, 1937, RENAUD, 1938; SINOTO & YUASA, 1941; SUBRAMANIAM 1946; LEVAN, 1946, 1947; DELAMATER, 1950; LIETZ, 1951; MUNDKUR, 1954. WINGE & ROBERTS 1954.
CENTROSOME OUTSIDE THE NUCLEAR VACUOLE: LINDEGREN ET AL., 1946-54.
SPINDLE WITH CHROMATIN INSIDE THE VACUOLE: LINDEGREN ET AL, 1956.
NUCLEAR VACUOLE: LINDEGREN ET AL. 1946-56
VACUOLE: BRANDT, 1941; SUBRAMANIAM, 1946, 1952; LIETZ, 1951; MUNDKUR, 1954.
VACUOLE: GUILLIERMOND, 1902, 1904, 1920, 1941.
NUCLEAR VACUOLE: WAGER, 1898, 1910.
NUCLEAR VACUOLE: JANSSENS & LEBLANC, 1898.
VACUOLE: 1844-1898.
CENTRAL VOLUTIN GRANULE: WAGER, 1910; NUCLEOLUS: LINDEGREN, 1956.
VACUOLAR MEMBRANE PRESUMED NAGEL, 1946; LINDEGREN, 1945-1956.
VACUOLAR MEMBRANE SEEN AS A LUMINOUS BORDER UNDER DARK GROUND ILLUMINATION. GUILLIERMOND, 1941.
IMPERMANENT: WAGER, 1898, 1910; BRANDT, 1941. DOES NOT SHOW CONTINUITY THROUGH SPORE: WAGER, 1898, 1910; GUILLIERMOND, 1920; LIETZ, 1951; WIDRA & DELAMATER, 1955.
PERMANENT CELL ORGANELLE. SHOWS CONTINUITY BY DIVIDING EITHER TO THE NUCLEI OF SPORES: WAGER, 1898, 1910; GUILLIERMOND, 1920; WINGE & LAUSTSEN, 1937; BADIAN, 1937; LIETZ, 1951.

Pictographic Summary 1

PICTOGRAPHIC SUMMARY 1.

Reconciliation of the Differing Views

In such a dilemma the obvious procedure is to carry out an extensive search of several species and strains of yeasts to see whether a nucleus having a conventional structure could be observed in living vegetative cells under some physiological conditions. The choice of vegetative cells is suggested by the facility with which they could be handled. A search under varying physiological conditions is necessitated by the doubt whether the high glycogen and RNA content at some stages of the growth cycle may not be responsible for the invisibility of the nucleus. The resting nucleus was the objective because an accurate knowledge of its structure is a prerequisite for attempts at elucidation of its behaviour during budding and spore formation.

Recent Advances

An extensive search carried out in this laboratory enabled the demonstration side by side of the vacuole and the nucleus in living vegetative cells of three

strains of *Saccharomyces cerevisiæ* (Royan and Subramaniam, 1956; Royan, 1956 *a*, *b*, *c*, 1958 *a*, *b*, *c*; Aswathanarayana and Subramaniam, 1958; Subramaniam, Royan, Thyagarajan, Aswathanarayana and Subramanyam, 1959), one of *Saccharomyces bayanus* (Aswathanarayana, unpublished) and in the living vegetative cells and zygotes of *Saccharomyces carlsbergensis* (Thyagarajan and Subramaniam, 1957 *a*, *b*; Thyagarajan, 1959). To observe the nucleus, the cells have to be aged in the malt wort in which they had grown. The nucleus becomes visible only when the glycogen disappears from the cells and the cytoplasm loses its intense basophilia (Royan, 1958 *b*). During the process of aging, the vacuolar membrane becomes well defined (Aswathanarayana, 1956 *a*, *b*) and the nucleus appears vesicular with a nuclear membrane enclosing formed structures (Royan, 1956 *a*). Often, the intra-nuclear structures appear plastered on to the nuclear membrane, which in consequence presents an irregularly thickened appearance.

Evidence that the nucleus is an extra-vacuolar structure is available from living cells. Under dark ground illumination the nuclear and vacuolar membranes delimit them from each other (Royan, 1956 *b*, 1958 *b*). When the cells are stimulated with fresh media the vacuoles disappear from view (Aswathanarayana, 1958). During the earlier stages of stimulation the encroaching cytoplasm separated the nucleus from the vacuole (Royan, 1956 *c*). Differential staining of the vacuolar membrane is difficult. But rare instances were available where the vacuolar membrane separated from the cytoplasm during the processing of the hæmatoxylin stained smears to make them permanent (Royan, 1958 *b*). In such cells the nucleus remained in the cytoplasm while the contracted but intact vacuolar membrane was seen lying free inside the vacuolar area (Subramaniam *et al.*, 1959).

When cells with visible nuclei are stained vitally with a proper dilution of neutral red, the vacuole alone has an affinity for the dye indicating that the nucleus and the vacuole are unrelated structures (Thyagarajan, 1958 *a*, *b*). During the segregation of neutral red by the vacuole, the vacuolar membrane was occasionally coloured by the dye.

The identification of the intra-nuclear structures into distinct categories necessitates fixation and staining (Photo 1). Since the vacuole has loomed large in discussions on the yeast nucleus, the fixative chosen should give a life-like preservation of the cell organelles. Most of the fixatives distort or destroy the vacuole. When preservation of one organelle is not exact, there would be legitimate doubts whether the same may not be the case with the others also. The situation becomes complicated when the nucleus is not visible in the living cells and the fixative distorts the prominent vacuole

(Ganesan and Swaminathan, 1958). An evaluation of the action of fixatives using cells with visible nuclei as the standard was therefore rendered imperative.

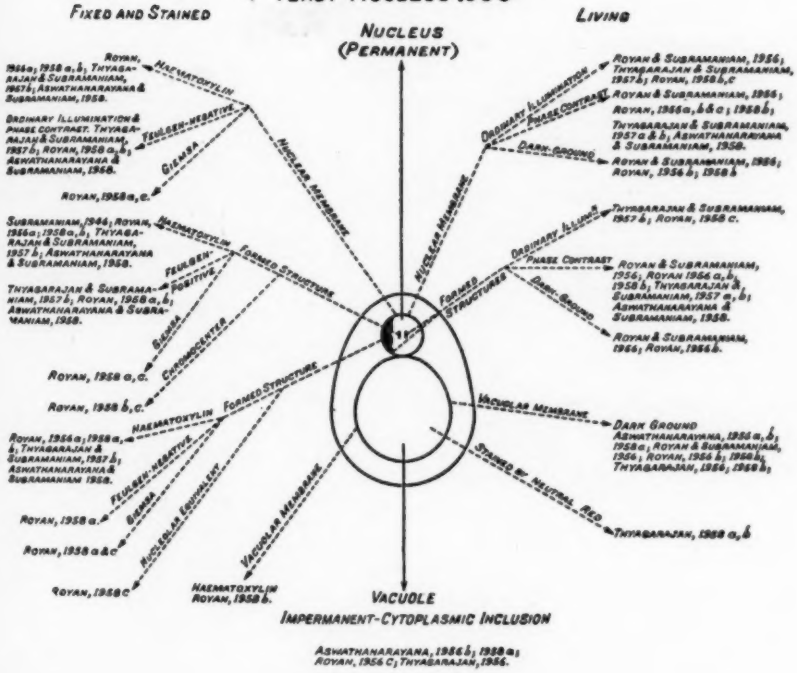
Iodine formaldehyde acetic solution when used in proper dilution gives a life-like preservation (Royan, 1956 *a*, 1958 *a*; Thyagarajan and Subramaniam, 1957 *b*; Aswathanarayana and Subramaniam, 1958). Correct fixation is but the first step in the analysis. Heidenhain's hæmatoxylin reveals all the structures visible in the living nuclei. The vacuole was bereft of stainable structures. When the cells are stained by the Feulgen technique, the stained region was seen to occupy only a portion of the nucleus. To determine the orientation of the stained region with reference to the nuclear membrane, examination under phase contrast was often necessary.

The identification of the Feulgen positive area as the whole nucleus on the assumption that the DNA is uniformly dispersed in the nuclear matrix, and the corollary that the small size of the nucleus (*ca* 0·5 μ) precludes any possibility of studying the yeast chromosomes (Winge and Roberts, 1954 *a*, *b*) find little support in the relatively large size of the nucleus (*ca* 2·0 μ) in living cells and the absence of any uniform staining of its matrix by the Feulgen stain. In fact, intra-nuclear structures could be clearly distinguished from bi-nucleate stages (Royan, 1958 *b*). The vacuole was Feulgen negative and hence is only a cytoplasmic inclusion.

A comparison of hæmatoxylin and Feulgen preparations showed that the nuclei contain Feulgen positive as well as Feulgen negative structures. The Feulgen positive areas are the chromocenters while the Feulgen negative structures are the equivalents of the nucleoli of higher organisms. The Giemsa stain has recently come into vogue in the study of the yeast nucleus. The general procedure is to stain the fixed smears after hydrolysis. The picture obtained is intermediate between hæmatoxylin and Feulgen preparations (Royan, 1958 *b*). But if unhydrolysed smears are stained with Giemsa, the chromocenters could be distinguished from the nucleolar equivalents by the differences in their colour (Royan, 1958 *c*; Subramaniam *et al.*, 1959).

The recent advances in our knowledge of the yeast nucleus based on a strict comparison of living with stained preparations would emphasize that the nucleus of yeast has a conventional vesicular structure (Subramaniam *et al.*, 1959) with a nuclear membrane, chromocenters and nucleolar equivalents (*Pictographic Summary II*, 1956).

PICTOGRAPHIC SUMMARY 2.



Pictographic Summary 2

SUMMARY

The disagreements on the question whether the nucleus of yeast is an organelle unrelated to the vacuole or whether it is the vacoule itself (*Pictographic Summary I*, 1844–1956) is only a corollary to the limitations of fixation and staining procedures carried out on cells in which the former was invisible. A reconciliation is possible if investigations are confined to cells in which both the organelles are visible. The extra-vacuolar nucleus has a conventional vesicular structure with a nuclear membrane, chromocenters and nucleolar equivalents. The vacuole, which has a limiting membrane in older cells, is Feulgen negative and segregates neutral red in living cells. It is therefore a cytoplasmic inclusion. A strict comparison of living with stained preparations is presented in *Pictographic Summary II*, 1956.

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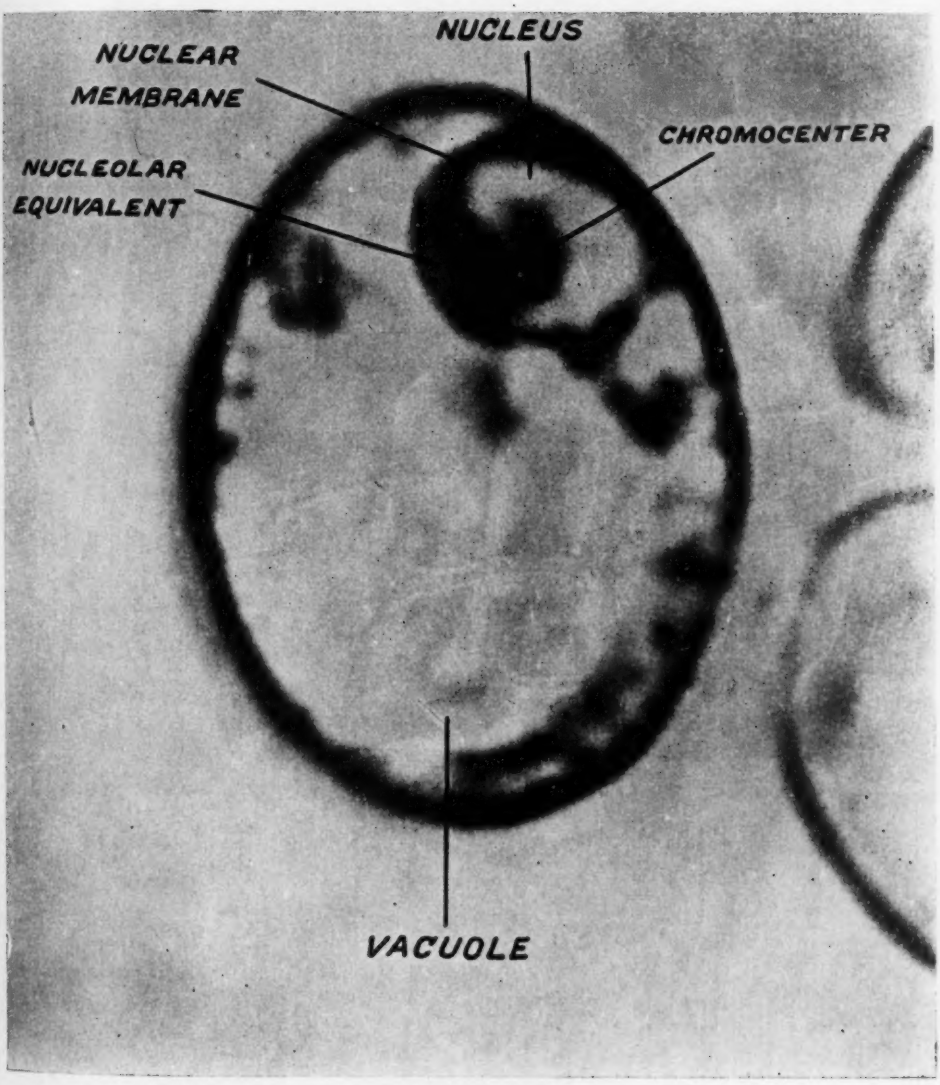

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OBSERVATIONS ON THE OSSIFICATION CENTRES OF *TRICHOPODUS TRICHOPTERUS* (PALL.)

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Introduction

The study of the ossification centres in fish skulls has been practically neglected in India. Hence, the present investigation was undertaken to examine the centres of ossification in the skull of *Trichopodus trichopterus* (Pall.), a freshwater fish, belonging to the family Anabantidæ. The development of the chondrocranium of the same fish has been worked out by the authors (1957).

Material and Methods

The fish was bred in the laboratory and the successive stages of fish-fry were fixed in acetic alcohol, and 3–5% formalin. The serial sections of different stages were cut at 10 micra and stained with Delafield's hæmatoxylin. The stages in which the bones had appeared were decalcified before sectioning. Alizarine transparencies of the various stages were made for noting the ossification centres.

Observations

Stage 1 (*Total length* 2·5 *mm.*).—The nasal sacs and a distinct ventral mouth have appeared. Some of the chondrocranial cartilages are laid down in this stage but there is no trace of any centre of ossification.

Stage 2 (*Total length* 4·0 *mm.*) (Fig. 1, *a–d*).—The ethmoid region does not show any ossification in this stage. In the orbito-sphenoidal region an elongated parasphenoid is noticed. In a lateral view the parasphenoid (*pasp*) shows a characteristic dorsal flexure and extends ventrally to the trabecula communis. In the auditory region the ossification centres of the pro-otic and sphenotic are seen. The pro-otic (*pro*) is large and the sphenotic (*sph*) very small. These are perichondral ossifications. In the occipital region, the basioccipital (*boc*) and exoccipitals (*exo*) are developed as perichondral ossifications.

The premaxilla (*pmx*) and the maxilla (*mx*) have appeared as thin, elongated dermal ossifications. The maxilla is slightly longer than the pre-

maxilla. The palatine and the different pterygoid ossifications have not yet appeared. The quadrate (*qu*) appears as a small ossification centre which is in the form of a triangular bony piece with a posteriorly directed process, reaching the anterior end of the symplectic. In the lower jaw the dentary (*den*) develops as a dermal ossification and anteriorly bears a few dermal denticles. A slender perichondral ossification centre is noticed behind the dentary and it is the articular (*ar*). Behind this is seen a very small centre of ossification of the retroarticular (*rar*).

In the hyoid arch the following centres of ossification are noticed—hyomandibular, symplectic, epi- and cerato-hyals and basihyal. The hyomandibular (*hym*) is being ossified and it shows the characteristic foramen (*fh*) for the exit of the hyomandibular branch of the facial. Its anterior and

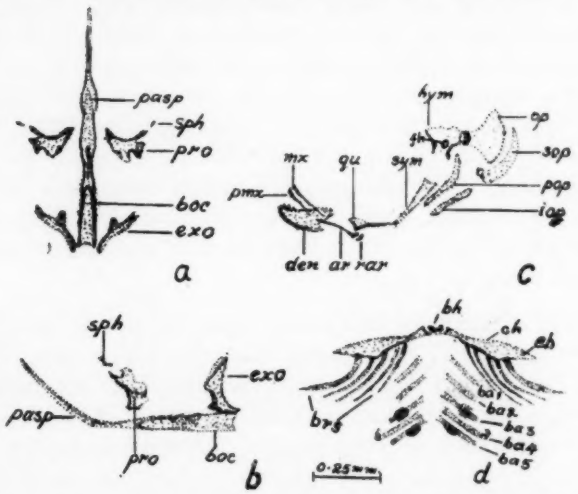

Fig. 1. (*a*) Dorsal aspect of the cranium of *Trichopodus trichopterus* (Pall.), 4 mm. long, showing ossification centres. The visceral arches are not shown.

(*b*) Lateral aspect of the same.

(*c*) Lateral, outer view of the jaws, hyopalate and opercular regions of *T. trichopterus*, showing ossification centres.

(*d*) Ventral view of the hyobranchial skeleton of *T. trichopterus*. The hyomandibular and symplectic are not shown.

posterior margins are better ossified. The symplectic (*sym*) appears as a conical perichondral ossification in the lower part of the hyosymplectic cartilage. The epi- and cerato-hyals (*eh* and *ch*) are uniformly ossified, and are not yet separated from each other. They together bear five characteristic

branchiostegal rays which are purely dermal structures. A small perichondral ossification gives rise to the basihyal (*bh*) situated between the two ceratohyals.

There appear four centres of dermal ossification in the opercular region. The preopercular (*pop*) seen behind the symplectic is an elongated centre of ossification. The opercular (*op*) is large and is already attached to the hyomandibular. The subopercular (*sop*) and interopercular (*iop*) are small.

No centre of ossification is seen in the region of the basibranchials. The ossification centres of the first four ceratobranchials (*cb* 1–4) and of the pharyngeum inferius (*cb* 5) along with its dentigerous pad have appeared.

Stage 3 (*Total length* 6·0 *mm.*).—As seen from the transverse sections the centres of ossification are better developed in this stage. In the sphenoidal region the frontals and the parietals have appeared. In the auditory region the epiotic and the pterotic make their appearance as perichondral ossifications. The pro-otic is better ossified in this stage, and the cartilage of the lateral and the anterior basicapsular commissures is ossified, forming a part of the pro-otic bone. The pro-otic exhibits a foramen for the passage of the VII cranial nerve. The sphenotic has slightly increased in size. The synotic tectum is being ossified perichondrally. In front of it is seen a dermal ossification, covering the brain. These two centres of ossification go to form the supraoccipital bone.

The premaxilla develops the small median ascending process, extending over the ethmoid cartilage. Palatine has made its appearance. It is the autopalatine and the ossification starts on the ventral side of the cartilaginous pterygoid process. The ectopterygoid also appears as a single centre of ossification. The ceratohyal is better ossified and the hypohyal is making its appearance in front of it. The basihyal is noticed as a ring-like perichondral ossification around the basihyal cartilage. The parahyoid appears as a small dermal ossification below the common copula but is not connected to it.

Stage 4 (*Total length* 7·0 *mm.*) (Fig. 2, *a–c*).—In the ethmoid region the lateral ethmoids (*let*) appear as small perichondral ossifications in the laminæ orbitonasales. The vomer (*vo*) appears as a small centre of ossification at the tip of the parasphenoid. The nasals are just appearing. In the sphenoidal region the frontals (*fr*) have increased in size and are posteriorly joined to the sphenotics (*sph*). The parietals (*p*) are better ossified along their anterior margin. The parasphenoid (*pasp*) has developed the lateral processes, which do not reach the pro-otics in this stage. In the auditory region all the four

otic bones of the adult fish are seen and the pro-otic (*pro*) is the largest. In the occipital region the basioccipital (*boc*) is better developed and is anteriorly produced into two processes. The supraoccipital (*so*) still exhibits its two centres of ossification, the anterior one situated in front of the parietals and the posterior near the epiotics.

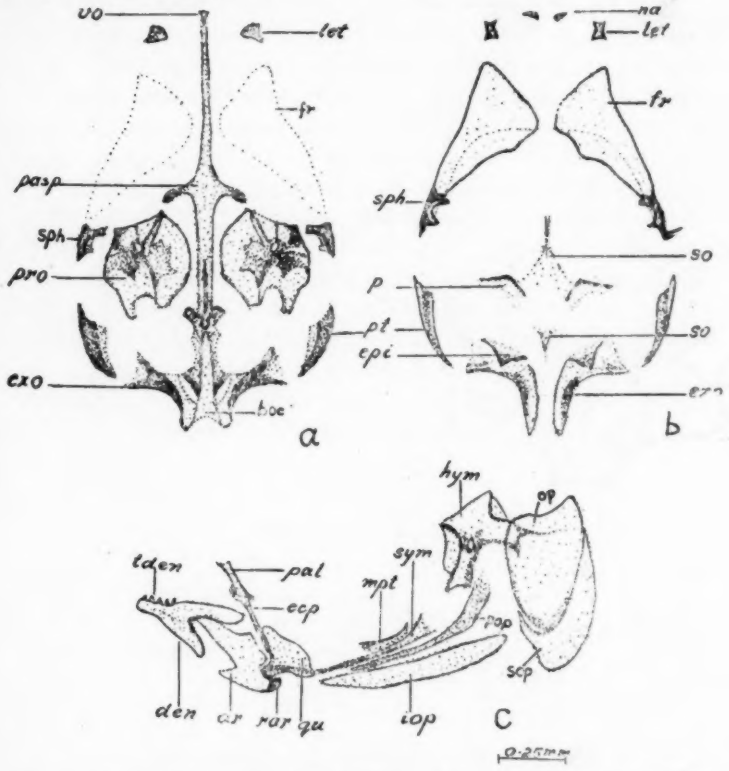

Fig. 2. (*a*) Ventral view of the cranium of *T. trichopterus*, 7 mm. long, showing ossification centres. The visceral arches are not shown.

(*b*) Dorsal view of the same.

(*c*) Lateral view of the jaw, hyopalate and opercular regions of the same, showing ossification centres.

The metapterygoid (*mpt*) appears as an elongated centre of ossification situated close to the symplectic. The stylohyal appears as a small perichondral ossification and is ventrally united with the epihyal. The epihyal and the ceratohyal are differentiated. The basihyal has developed into an elongated cylindrical piece. The preopercular (*pop*) has increased in size

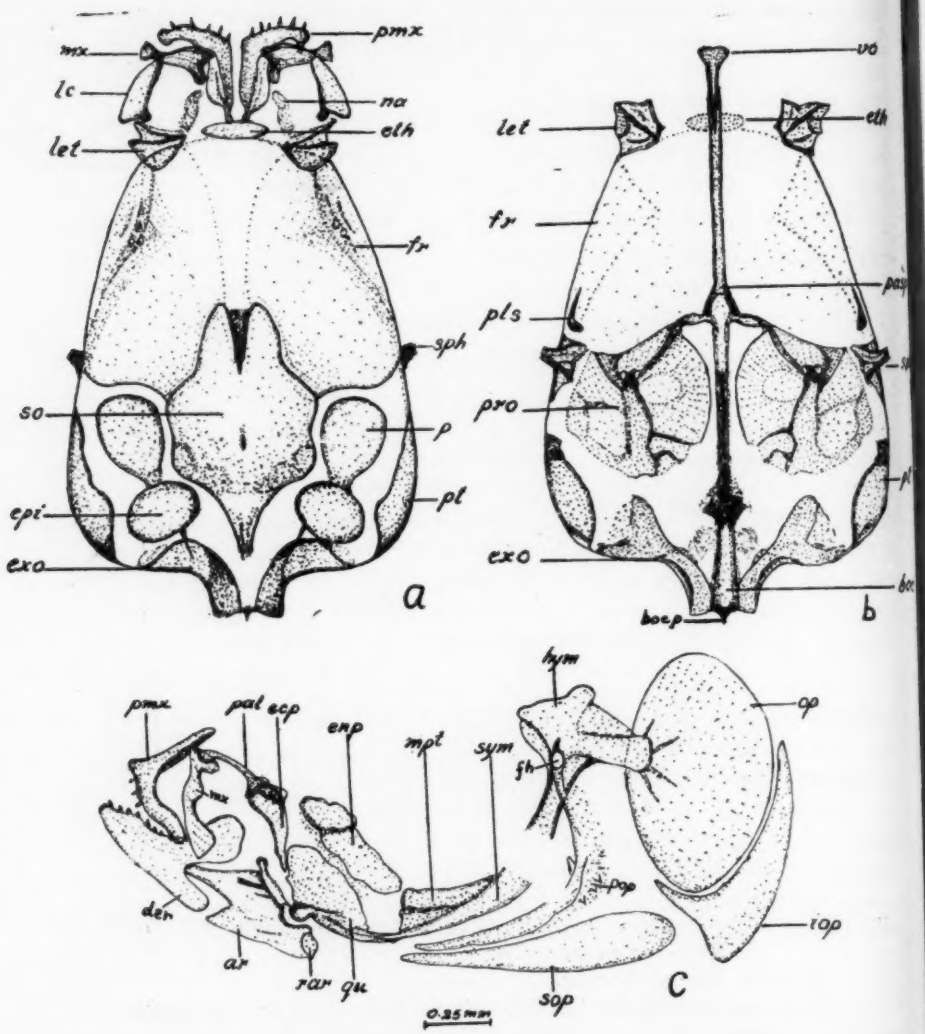

FIG. 3. (*a*) Dorsal view of the cranium of *T. trichopterus*, 10 mm. long, showing the ossification centres. The visceral arches are not shown.

(*b*) Ventral view of the same.

(*c*) Lateral view of the jaws, hyopalate and opercular regions of the same, showing the ossification centres.

and bears a few denticles. Three centres of ossification are seen in the basibranchial region. The anterior centre is in contact with the basihyal. The middle gives attachment to the first pair of branchial arches and the posterior to the second and third. The first three pairs of hypobranchials are being ossified. The three pharyngobranchials have appeared.

Stage 5 (*Total length* 10·0 *mm.*) (Figs. 3, *a–c* and 4).—In addition to the centres of ossification already described, the ethmoid region shows the median ethmoid (*eth*) for the first time. It appears as an ovoid centre of perichondral ossification, situated dorsally between the two lateral ethmoids. The dermal centre of ossification of the nasal (*na*) is better represented. The lachrymal (*lc*) or the first orbital is well developed. The remaining orbital bones are appearing. The frontals and the parietals have increased in size. The pleurosphenoid (*pls*) appears as a small centre of ossification situated in front of the sphenotic. The parasphenoid is very well represented, exhibiting the characteristic features of the adult skull. Its lateral processes meet the pro-otics. The epiotic (*epi*) exhibits its characteristic posterior lamella. The occipital bones are well represented. The basioccipital (*boc*) has developed posteriorly a small blunt process, as seen in the adult skull. The centres of ossification of the supratemporal and the post-temporal have appeared.

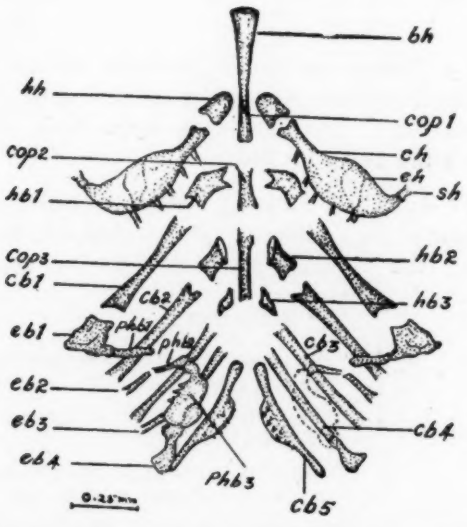

FIG. 4. Dorsal aspect of the hyobranchial skeleton of *T. trichopterus*, 10 mm. long, showing the ossification centres. The hyomandibular and the symplectic are not shown.

The entopterygoid (*enp*) appears for the first time and is situated dorsal to the quadrate. The anterior part of the symplectic (*sym*) is wedged into the quadrate (*qu*). In the branchial skeleton, the four epibranchials (*eb* 1–4) are added in this stage. The remaining branchial elements increase in size. The first epibranchial is large and is taking up the characteristic shape found in the adult skull.

SUMMARY

The ossification centres in the skull of *T. trichopterus* have been studied in five stages.

(*a*) In stage 1 (2·5 mm.), the centres of ossification have not yet appeared.

(*b*) In stage 2 (4 mm.), the parasphenoid, the pro-otics, the exoccipitals and the basioccipital have appeared in the cranium. The premaxilla, maxilla, quadrate, dentary, articular and retroarticular are seen in the jaws and palate region. In the hyoid arch and opercular region all the bones are seen excepting the stylohyal, the hypohyal and the parahyoid. In the branchial region, centres of ossification of the ceratobranchials, and the pharyngeum inferius are noticed.

(*c*) In stage 3 (6 mm.) the frontals, parietals, pterotics, epiotics and supraoccipital are added to the cranium. The palatines (autopalatines), ectopterygoids, hypohyals and parahyoid are added to the visceral skeleton in this stage.

(*d*) In stage 4 (7 mm.) the lateral ethmoids, vomers and nasals have appeared. The remaining centres are—the metapterygoid, the stylohyal and the three basibranchials. The first three hypobranchials and the three pharyngobranchials are being ossified.

(*e*) In stage 5 (10 mm.) the following centres of ossification have appeared —The median ethmoid, the orbitals, the pleurosphenoids, the supratemporals, the post-temporals, the entopterygoids, and the four epibranchials.

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Key to Abbreviations

ar	.. articular	*lc*	.. lachrymal or first orbital
bh	.. basihyal	*let*	.. lateral ethmoid
bs 1–5	.. branchial arches 1–5	*mpt*	.. metapterygoid
boc	.. basioccipital	*mx*	.. maxilla
bocp	.. basioccipital process	*na*	.. nasal
brs	.. branchiostegal rays	*op*	.. opercular
cb 1–5	.. ceratobranchials 1–5	*p*	.. parietal
ch	.. ceratohyal	*pal*	.. palatine
cop 1–3	.. basibranchials or copulæ 1–3	*pasp*	.. parasphenoid
den	.. dentary	*phb* 1–3	.. pharyngobranchials 1–3
eb 1–4	.. epibranchials 1–4	*pls*	.. pleurosphenoid
ecp	.. ectopterygoid	*pmx*	.. premaxilla
eh	.. epihyal	*pop*	.. preopercular
enp	.. entopterygoid	*pro*	.. pro-otic
epi	.. epiotic	*pt*	.. pterotic
eth	.. median ethmoid	*qu*	.. quadrate
exo	.. exoccipital	*rar*	.. retroarticular
fh	.. foramen for the hyomandibularis branch of the facial	*sh*	.. stylohyal
		so	.. supraoccipital
fmg	.. foramen magnum	*sop*	.. subopercular
fr	.. frontal	*sph*	.. sphenotic
hb 1–3	.. hypobranchials 1–3	*sym*	.. symplectic
hh	.. hypohyal	*tden*	.. teeth or denticles on dentary
hym	.. hyomandibular	*vo*	.. vomer
iop	.. interopercular		

THREE NEW SPECIES OF BRACONIDAE FROM INDIA

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Received June 8, 1959

Introduction

The hymenopterous family Braconidæ includes many genera and species which are parasitic on various crop pests and are, therefore, of interest to the economic entomologist. However, the taxonomy of the Indian forms of Braconidæ has not been intensively studied so far, apart from the excellent contributions on particular groups made by Nees, Wilkinson, Ramakrishna Ayyar and Bhatnagar. The braconid fauna of this sub-continent is rich and varied and its study should prove to be a rewarding one.

The present contribution contains descriptions of three new species belonging to three braconid genera: *Aphidius* (*Lysiphlebus*) *delhiensis* n. sp., *Microgaster adisuræ* n. sp. and *Philomacroploea cameroni* n. sp.

Aphidius (*Lysiphlebus*) *delhiensis* n. sp.

Female.—Length 1·40–1·60 mm. Head 0·30–0·35 mm. wide, facial line 0·19–0·23 mm., clypeoantennal line 0·07–0·08 mm., interocular line 0·13–0·19 mm., transfacial line 0·11–0·17 mm. Antennæ 13 segmented (none of the 44 females examined had less than 13), first flagellar segment 0·085–0·10 mm., second flagellar segment 0·081–0·090 mm.

Thorax smooth, 0·35–0·44 mm. wide at the tegulæ; notaulices weakly developed anteriorly. Stigma 0·250–0·285 mm. long, 0·71–0·89 mm. wide; metacarpus 0·214–0·250 mm.; first abscissa of radius 0·089–0·125 mm.; second abscissa of radius 0·071–0·09 mm.; cubital cross-vein 0·053–0·063 mm.

Petiole 0·018–0·0214 mm. long, 0·09 mm. wide at the spiracles. Genitalia illustrated on Fig. 1, 3.

Coloration.—Head, especially eyes, deep-brown; mouth parts pale-yellow; antennæ brown, basal portion lighter than apical. Thorax brown, prothorax almost as dark-brown as head; pleural region lighter than dorsum.

Wings hyaline, veins and stigma brown. Petiole and first abdominal segment testaceous or yellowish, rest of abdomen dark-brown. Coxæ and femora testaceous, tibiæ and tarsi brownish.

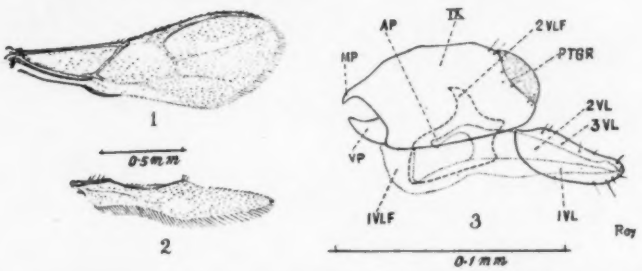

FIG. 1. 1. Forewing of *Lysiphlebus delhiensis* n. sp., ♀. 2. Hindwing of *Lysiphlebus delhiensis* n. sp., ♀. 3. Genitalia of *Lysiphlebus delhiensis* n. sp., ♀. AP, Anterior prong of the second valvulæ; MP, Median prong of the ninth abdominal tergite; *ix*, Ninth abdominal tergite; PTGR, Proctiger; 1 VL, 2 VL, 3 VL, First, second and third valvulæ; 1 VLF, 2 VLF, First and second valvifer; VP, Ventral prong of the ninth abdominal tergite.

Male.—Antenna 14–15 segmented, majority with 15 segments (45 out of 55 specimens examined); two had 14 segments in one antenna and 15 segments in the other.

Coloration.—Similar to that of the female except that the pleural region of the thorax is generally darker.

Host.—Longiunguis sacchari (Zehnt.) on sugarcane.

Locality.—Indian Agricultural Research Institute, New Delhi. Coll.: A. K. Sharma, October–November 1957.

This species has been described from a large number of individuals obtained from parasitised aphids collected from the sugarcane plants. The holotype female and 4 paratypes (2 males, 2 females), all on card-strips, are deposited in the National Pusa Insect Collection of I.A.R.I., while certain paratypes are in the possession of the authors.

Of the known species of the subgenus *Lysiphlebus* Foerster, this species comes closest to *testaceipes* (Cresson) from which it differs in various characters, *e.g.*, head width, venation, the female genitalia and coloration.

Microgaster adisuræ n. sp.

Female.—Head somewhat punctate, especially on the vertex; distance between posterior ocelli slightly less than the distance between the ocellus and the eye of the same side. Antennæ 18 segmented, longer than the entire body.

Thorax weakly punctate, mesopleural sulcus shallow, short, broad. Areola practically three-sided, breadth of stigma greater than the length of the first abscissa of the radius; stigma longer than the metacarp (Fig. 2, 1). Hind coxa smooth; shorter hind tibial spur 0·117 mm., longer hind tibial spur 0·135 mm.; basal segment of hind tarsus 3·303 mm. Propodeum markedly reticulate, without a median longitudinal carinæ (Fig. 2, 2).

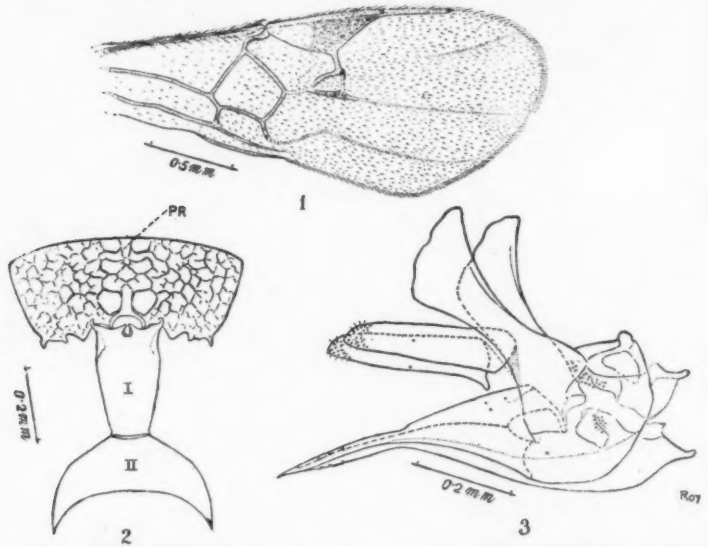

FIG. 2. 1. Forewing of *Microgaster adisuræ* n. sp., ♀. 2. Propodeum, first and second abdominal tergites of *Microgaster adisuræ* n. sp., ♀. 3. Genitalia of *Microgaster adisuræ* n. sp., ♀. I, II., First and second abdominal tergites; PR, Propodeum.

First tergite of the abdomen smooth, about twice as long as broad; second tergite about ⅓rd as long as its breadth at the posterior corners (Fig. 2, 2). Ovipositor sheaths without paired processes (Fig. 2, 2).

Coloration.—Head, thorax dark; propodeum dark; basal tergites and sternites of abdomen testaceous yellow, rest dark but slightly lighter than the thorax; hind coxæ dark above, somewhat lighter below; hind tibiæ brownish. Wings slightly infumated in the apical half, vein deep-brown, stigma dark in the apical ⅔rds, the basal ⅓rd hyaline.

Male.—Antennæ 20 segmented; coloration similar to that of female except in being slightly darker generally and in having the abdomen entirely dark.

The female genitalia of this species are particularly interesting and serve as its most distinctive character.

Host.—Caterpillars of *Adisura atkinsoni* Moore, the lab-lab pod borer collected at Bangalore, South India, by B. R. Subba Rao, 1957.

This species is being described from a holotype female and an allotype male, deposited in the National Pusa Insect Collection, I.A.R.I., and a male and a female paratype each dissected on slides, in possession of the authors.

A perusal of literature shows that this is the first record of a species of *Microgaster* parasitising *Adisura atkinsoni* Moore. Further, the characters of this species are such that it is difficult to compare it with any known species of *Microgaster*.

Philomacroplæa Cameron

Ashmead (1900) erected the genus *Chelonogastra* with *C. kæbeli* Ashm. from Japan as its single representative. The description of *kæbeli* as well as of another species *pleuralis* was, however, published by Ashmead only in 1906.

In 1906, Cameron established the genus *Philomacroplæa* and described a single species under it, *viz.*, *P. basimacula* from Ceylon.

Ramakrishna Ayyar (1928) synonymised *Chelonogastra* Ashmead with *Philomacroplæa* Cameron, retaining *Chelonogastra* for reasons of priority.

However, two of Ashmead's paratypes in the U.S. National Museum and the genotype of Cameron's *Philomacroplæa* in the British Museum were subsequently examined by Waterston (1929), who came to the conclusion that Ramakrishna Ayyar's view was incorrect and that *Chelonogastra* and *Philomacroplæa* are distinctly different genera, with clearly distinguishable characters.

Recently the authors had the opportunity of examining certain museum specimens which appeared to belong to *Chelonogastra*. However, a more careful examination revealed that these specimens really belong to *Philomacroplæa* and that the distinguishing characters of this genus as enunciated by Waterston were quite sound, notwithstanding the fact that he had examined only a single specimen, *i.e.*, the genotype of Cameron's. But the specimens examined by us do not appear to be the same as *P. basimacula* Cam., and apparently belong to a new species, which is being described as follows:—

Philomacroplæa cameroni n. sp.

Female.—About 3·5–3·60 mm. Head slightly narrower than the thorax, twice as broad as long. Antennæ longer than the head and thorax combined, 23 segmented. Eyes large, non-pubescent; ocelli arranged in an equilateral triangle, ocellar region prominently raised.

Thorax as shown in Fig. 3, 1 propodeum with a very faint, low, median longitudinal keel or carina indicated only anteriorly 2nd abscissa of the radius almost equal to the 1st transverse cubital vein (Fig. 3, 1).

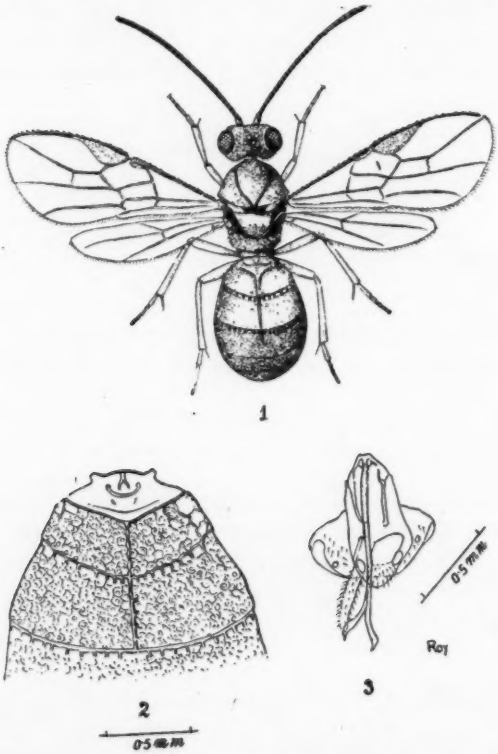

FIG. 3. *Philomacroplæa cameroni* n. sp., ♀. 2. First, second and third abdominal tergites of *Philomacroplæa cameroni* n. sp., ♀. 3. Genitalia of *Philomacroplæa cameroni* n. sp., ♀.

First tergite of the broad, rounded abdomen, depressed, with a strong ridge at the posterior margin. Second and third tergites with a well marked median longitudinal keel and umbilicate puncturation; fourth, fifth and sixth segments without median keel but possessing umbilicate puncturation.

Posterior margins of the second and third segments provided with blunt prolongations, the longitudinal carina of these segments also gives off similar projections (Fig. 3, 2). Ovipositor short, about 1/5 the length of the abdomen (Fig. 3, 3).

Entire body covered with a fine dense pubescence except for the bare eyes; a tuft of especially long pubescence present in incised middle of the last abdominal segment whence issues the ovipositor.

Coloration.—Antennæ, head and thorax brown, eyes and ocellar area deep-brown. First abdominal segment completely dark-brown, middle of second and third segments much lighter, lateral margins dark-brown; fourth, fifth and sixth segments entirely dark-brown; ovipositor testaceous.

Male.—Essentially similar to the female, but not so stoutly built. Antennæ 26 segmented, more slender. Abdomen less dark-brown than in the female.

Described from a holotype female and an allotype male and 3 paratypes (1 male and 2 females).

Host.—" Pupæ of butterfly on *Calotropis*, " Gwalior, 1956 (presumably *Danais* sp.).

This species differs from the only other described species,* of the genus, *viz.*, *Philomacroplæa basimacula* Cameron in the following characters: the weakly developed median carina of the propodeum, the depressed first tergite of the abdomen, the coloration of the abdomen and the peculiar prolongations of the posterior margins of the second and third segments.

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* Ramakrishna Ayyar (1928) described a species *Chelonogastra trifasciata* which might have been a species of *Philomacroplæa*. However, the description—as the author himself states—was only a provisional one and rather incomplete; no figures or diagrams were given, either. Even then, the coloration was quite different especially in the presence of three dark stripes on the mesothorax.

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EXPERIMENTAL CUTANEOUS LESIONS IN THE ALBINO RAT

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A peculiar, dry, earthy coloration of the skin has been mentioned in association with ocular symptoms by most of the workers on keratomalacia. A detailed clinical description of the changes in the skin in cases of keratomalacia in adults was given by Pillat (1929 *a* and *b*), emphasising that keratomalacia is a 'system disease' of the ectodermal tissues, manifesting symptoms not only in the eye but also in several other parts of the body. Infections of the skin, such as pustular eruptions, furunculosis, abscesses, impetigo, acute bed-sores (in babies), etc., have often been associated with keratomalacia.

Besides the abovementioned changes, a follicular keratosis of the skin has been described occurring with xerophthalmia and keratomalacia by Frazier and Hu (1930, 1931) in China, Loewenthal (1933 *a* and *b*) in East Africa, Nicholls (1933) in Ceylon and Radhakrishna Rao (1937 *a* and *b*) in India.

In animals, especially in rats, several workers have tried to produce uncomplicated vitamin A deficiency and proved that dietary deficiency of vitamin A alone was responsible in producing metaplasia of columnar, cuboidal and transitional epithelia to the squamous keratinizing type in some organs. Previous literature reveals that there is no confusion regarding the primary histologic alterations and resulting lesions of the eye, the paraocular glands and the respiratory, gastrointestinal and genitourinary systems. However, the question of the cutaneous lesions in vitamin A deficiency in rats was until recently not very clear.

The proof that the histological changes occurring in rat's skin are in certain respects identical with those described in human beings in cases of 'phrynoderma' comes mainly from the work of Sullivan and Evans (1943) and Moult (1943). They have independently shown that keratotic plugs

may readily be induced in rats maintained on a diet deficient in vitamin A and that these plugs are identical with those occurring in human subjects.

Most of the workers cured cases of 'phrynoderma' by administering cod liver oil. The cure brought about by cod liver oil was attributed to its high vitamin A content. But the latest work on 'phrynoderma' has thrown some doubt on this hypothesis. Gopalan (1947) has treated cases of 'phrynoderma' by combined treatment with yeast extract and linseed oil, whilst Menon, Tulpule and Patwardhan (1950) have succeeded in curing 'phrynoderma' with gingelly oil.

A review of the literature reveals that very little attention has been paid to the detailed histological study of the skin lesions occurring in the fat deficiency syndrome. Only Williamson (1941) has shown that the epidermis of rats on fat-free diets becomes thicker and more differentiated than the normal ones.

However, after the completion of the present work undertaken in 1950, Ramlingswami and Sinclair (1953) have reported plugging of the follicular openings with dense compact layers of keratin and acanthosis of their lining epithelium in albino rats maintained on fat deficient diets.

The object of the present investigation was to elucidate the comparative functions of vitamin A and essential fatty acids in maintaining the epithelium of the skin of the rat in a normal state and to study the effects of deficiencies of these factors on the structure of the skin.

TABLE I

Composition of the basal diet and vitamin supplements

(Experiment 1)

Ingredients	Parts	Vitamin supplements per rat per day		
Casein (purified) ..	18	Vitamin D	10	U.S.P. units
Starch	32·5	Vitamin E	0·43	mg.
Sucrose	32·5	Vitamin A (Group I Control)	50	I.U.
Fat (Peanut oil)	5	Vitamin A (Group II)	5	I.U.
Osborne and Mendal's Salt Mixture No. 1 332	4	Vitamin A (Group III)	1	I.U.
		Vitamin A (Group IV)	0	I.U.
Dried Brewer's Yeast (purified)	8			

Experimental

The following experiments were planned to produce uncomplicated vitamin A and essential fatty acid deficiencies in rats maintained on a synthetic diet, with a view to study the clinical manifestations and histological changes occurring in the skin, in the complete absence of vitamin A, as well as when given at suboptimal levels.

Animals

Healthy, young albino rats of the Haffkine Institute inbred strain, with an average weight of 39 gm. were selected for Experiment No. 1. In all, 24 rats (12 males and 12 females) were distributed, as far as possible, with respect to weight, littermates and sex into four different groups. For the second experiment, 30 rats with an average weight of 26 gm. were divided in five different groups.

Diet

The composition of the basal diet is indicated in Tables I and II. Cornstarch, casein and yeast used in the diet were subjected to purification.

Table II

Composition of the basal diet and vitamin supplements

(Experiment 2)

Ingredients	Group I	Group II	Group III	Group IV	Group V
Casein (purified) ..	18	18	18	18	18
Starch (purified) ..	..	..	..	..	50
Sucrose ..	65	65	65	70	20
Fat					
(1) Peanut oil ..	5	..	..	..	..
(2) Lard ..	..	5	..	..	..
(3) Hydrogenated oil (Vanaspati) ..	..	..	5	..	..
Osborne and Mendal's Salt Mixture No. 1 332	4	4	4	4	4
Dried Brewer's Yeast (purified)	8	8	8	8	8
	100	100	100	100	100

Vitamin supplements per rat per day (Incorporated in the diet) for all groups

Vitamin A (Nonsaponifiable fraction of Shark Liver Oil) ..	100	I.U.
Vitamin D	10	U.S.P. Units
Vitamin E	0·43	mg.

The required amounts of vitamin A, D and E were administered to each rat orally in olive oil.

Experiment 1

The above diet was supplied *ad libitum* and a record of the daily food consumed by each rat was maintained. The experiment lasted for 101 days.

As the experiment progressed, animals on completely vitamin A-free diet showed marked emaciation. When they became moribund, they were sacrificed by ether anæsthesia. The corresponding animals from the other groups were also sacrificed for comparative study.

Skin pieces from different parts of the body were excised for histopathological study. As the structure of the skin from the different regions was observed to be of a similar nature, the findings of sections taken from the dorsal region are presented. In this region, the stratum corneum of the skin was found to be consisting of a thin layer of cells, hence changes occurring as a result of vitamin A deficiency, like hyperkeratinization, can very easily be detected and studied.

The skin pieces were fixed in 10% formal saline, and blocked in paraffin. The sections were cut to 6–7 μ in thickness. They were stained by Ehrlich's acid hæmatoxylin and eosin, and by Weigert's iron hæmatoxylin and Van Giesson's stains.

Results

Group I: (Control) 50 I.U. vitamin A per rat per day

Throughout the period of experiment (101 days) these animals appeared very healthy, active and steadily gained in weight, till they were sacrificed. Their average gain in weight was 187 gm.

Clinical and histological observations.—Examination of the skin did not reveal any abnormality (Fig. 2).

Group II: 5 I.U. vitamin A per rat per day

All the animals steadily gained in weight. Throughout the experiment the average gain in weight was 162 gm.

Clinical signs and symptoms.—All the rats apparently remained healthy and did not show any clinical signs or symptoms of vitamin A deficiency.

Histological observations.—Though none of the rats of this group showed any signs or symptoms of vitamin A deficiency clinically, the histological examination of the skin, in 3 cases out of 6 revealed changes typical of vitamin A deficiency.

1. *Epidermis.*—Three rats showed slight hyperkeratinization of the stratum corneum. All the other layers were normal.

2. *Corium.*—Normal.

3. *Sebaceous glands.*—Normal.

4. *Hair follicles.*—Few of the hair follicles showed slight dilatation and plugging of their orifices with keratinous material. This was observed in two cases where there was slight hyperkeratinization of the stratum corneum layer. The rest did not show any changes. There was a good amount of deposition of subcutaneous fat in all the animals.

Group III : 1 *I.U. vitamin A per rat per day*

The animals of this group, for the first 11 weeks, steadily gained in weight, the average gain in weight being 122 gm. The last three weeks showed slight decline in the weight curve.

Clinical signs and symptoms.—Coincident with the decline in weight all the animals showed signs and symptoms of vitamin A deficiency. In this group three rats developed typical xerophthalmia and other two rats showed only encrusted eyelids, photophobia and lacrymation. One did not show any eye changes throughout the period of experiment. The animals slowly became emaciated and their skin appeared loose.

Histological observations : 1. *Epidermis.*—The stratum corneum of all animals showed marked hyperkeratinization, as compared to the control rats of the group I. The keratin was present in loose lamellæ. Other layers were normal.

2. *Corium.*—Normal.

3. *Sebaceous glands.*—Normal.

4. *Hair follicles.*—Though there was general hyperkeratinization only in two animals, typical dilatation and plugging of hair follicles with loose keratinous material and slight atrophy of their lining epithelium was observed. In the remaining four cases, they appeared normal. Subcutaneous fat store was diminished (Fig. 3).

Group IV : 0 *I.U. vitamin A per rat per day*

For the first six weeks of the experiment all the animals steadily gained in weight, the gain being comparatively less than in the other groups. The total average gain in weight was 75 gm. After the period of initial six weeks, the weight started declining till they were sacrificed. After three weeks the food consumption was lower than that of the control group.

Clinical signs and symptoms.—Coincident with the decline in weight, symptoms of vitamin A deficiency developed. All the rats started showing signs of vitamin A deficiency between the 5th and 7th week of the experiment.

The first visible manifestation of vitamin A deficiency was a brownish encrustation on the eyelids. This was followed by the eyelids becoming puffy, the animals developing photophobia and later on lacrymation. Usually both eyes were involved at about the same time. Loss of lustre and dryness of cornea soon followed. Serosanguinous exudate was found to be present round about the eyes and the animals kept on rubbing the eyes as if it caused irritation. Thus, xerophthalmia of the eyes developed (Fig. 1). Then slowly the cornea became opaque and covered with pus and finally general inflammation of the eyeball set in (panophthalmitis). It became so acute that the whole eyeball degenerated leaving a cavity filled with a serosanguinous exudate.

In some rats swelling in the areas above the snouts was observed. Side by side, the skin became very loose probably due to the loss of subcutaneous fat. Hair became coarse and showed a tendency to easy epilation. The animals became highly emaciated, so much so their weight dropped from an average of 115 gm. to an average of 73 gm. Often their bodies were found covered with blood stains probably due to their rubbing the eyes which bled on slightest touch. The animals became humped and nervous. In two rats, there was denudation of the hair around the eyes. All animals were found sneezing and on post-mortem examination, showed inflammation and congestion of the lungs.

Histological observations : 1. *Epidermis.*—The chief pathological changes were found in the corneum layer. All the rats of this group showed excessive hyperkeratinization both follicular and diffuse. The keratin was present in loose thin wavy lamellæ. All the other layers appeared normal.

2. *Corium.*—In two rats, where there was extreme hyperkeratinization, slight lymphocytic infiltration of corium especially in the perifollicular region was observed. In the other four rats, the corium appeared normal.

3. *Sebaceous glands.*—These were present in all the sections but frequently, the glands present at the root of distended plugged hair follicles were found shrivelled up. The glands often showed decrease in the number of cells and their cytoplasm.

4. *Hair follicles.*—Marked changes were found in the hair follicles of this group of rats. The orifices of the hair follicles showed marked hyperkeratinization of the lining epithelium and, as a result, the funnels of the follicles were widened and were plugged by dense masses of horny tissue. The follicular plugs consisted of lamalle of flattened cornified cells in which there were no nuclei. In extreme cases, the lining of the hair follicles were found to be atrophic. Sections of broken hair were often seen in the substance of follicular plugs. There was marked reduction in the store of subcutaneous fat (Fig. 4).

Experiment 2

The duration of the experiment was 23 weeks. For the first 12 weeks, the animals were supplied food *ad libitum*. Within 8 weeks, the deficiency symptoms became evident. On being continued on the same diet, instead of becoming moribund they went into a chronic deficiency state. At this stage, it was found necessary to bring them in an acute deficiency state by inflicting some stress on the metabolism. This was done by restricting the diet to 6 gm. per rat per day for the rest of the duration of the experiment.

When they appeared highly emaciated, the animals were sacrificed by ether anesthesia. Corresponding animals from other groups were also sacrificed and the histopathology of skin studied as in the last experiment.

Results

Group I: 5% peanut oil

Throughout the experiment the animals appeared very healthy and active. They steadily gained in weight for the first 12 weeks and then maintained a plateau on the restricted food intake regime. Their average gain in weight was 120 gm. during the course of the experiment. Since the animals were kept on a restricted caloric diet after 12 weeks, their gain in weight is less compared to the rats of the control in the last experiment.

Clinical and histological observations.—Examination of the skin did not reveal any abnormality.

Group II: 5% lard

Throughout the experiment they appeared healthy and active. Their weight curve coincided with that of the control group. The total average gain in weight at the end of the experiment was 114 gm.

Clinical signs and symptoms.—Clinically, the animals appeared healthy, and did not show any macroscopic lesions throughout the experiment.

Histological observations.—Histological examination revealed slight changes in the epidermis, though clinically the animals did not show any signs and symptoms.

1. *Epidermis.*—Out of the six rats, two rats showed considerable amount of hyperkeratinization of the stratum corneum layer. Two other rats showed slight hyperkeratinization of the stratum corneum, whilst in the remaining two stratum corneum appeared to be normal. Other layers of the epidermis were normal.

2. *Corium.*—Normal.

3. *Sebaceous glands.*—Normal.

4. *Hair follicles.*—Though there was hyperkeratinization no plugging or dilatation of hair follicles was observed in any case. In all cases they were normal.

There was a good amount of subcutaneous fat deposition.

Group III : 5% hydrogenated oil

Throughout the period of experiment, their weight curve coincided with that of the control group. At the end of the experiment, the average gain in weight was 111 gm. Throughout the period of experiment, the animals appeared healthy and active.

Clinical signs and symptoms.—Clinically all the six rats appeared healthy and did not show any outward signs and symptoms.

Histological observations.—Histological study revealed following changes in the skin structure.

1. *Epidermis.*—All the rats showed hyperkeratinization of the stratum corneum layer as compared to the peanut oil group. In one case, it was severe whilst in other cases, it was moderate. Keratin was present in dense masses. All the other layers of the epidermis appeared normal.

2. *Corium.*—Normal.

3. *Sebaceous glands.*—Normal.

4. *Hair follicles.*—Four cases out of six showed dilatation and plugging of orifices of hair follicles, with dense keratinous masses. In the other two cases, they appeared normal.

There was a marked reduction in the subcutaneous fat store in all the rats (Fig. 6).

Group IV: Fat-free diet (sucrose)

For the first 5 weeks the average gain in body weight equalled that of the control group. After that, the gain in weight was very little, followed by loss in weight on restricted food intake. At the end of the experiment, the gain in weight averaged only 44 gm.

Clinical signs and symptoms.—For the first five weeks, all the animals appeared healthy. Then they slowly started showing the deficiency symptoms. In the 9th week, typical ringed tail condition was evident. The tip of the tail and paws, especially hind ones, were erythematous and scaly. Pinnæ of the ears also showed slight scaliness. There was scanty growth of hair on the abdominal and facial regions. At a very late stage of the disease, the abdominal region of two rats were found constantly covered with a yellowish sticky exudate. Post-mortem examination showed intestinal distention accompanied by loss of subcutaneous and visceral fat (Fig. 5).

Histological observations.—Histological study of the skin revealed the following changes.

1. *Epidermis.*—All the rats showed excessive hyperkeratinization of the stratum corneum layer. Keratin was present in dense masses. All the other layers appeared normal.

2. *Corium.*—Normal.

3. *Sebaceous glands.*—All animals showed normal structure.

4. *Hair follicles.*—Marked changes were found in the hair follicles of this group of rats. The orifices of the hair follicles showed marked hyperkeratinization of the lining epithelium, as a result, the funnels of the follicles were widened and plugged by dense masses of keratin. Sections of broken hairs were seen in the substance of follicular plugs.

There was complete loss of subcutaneous fatty tissue (Fig. 7).

Group IV: Fat-free diet (starch)

For the first 9 weeks, the average gain in body weight equalled that of the controlled group. After that, the gain in weight was less than that found in the first 3 groups, *viz.*, peanut oil, lard and hydrogenated oil respectively, but greater than in group IV, *i.e.*, those on fat-free diet containing 70% sucrose. The average gain in weight at the end of the experiment was 70 gm.

Clinical signs and symptoms.—Typical fat deficiency signs and symptoms were not observed in this group. For 10 weeks the animals appeared healthy. Then, they slowly started showing emaciation followed by slight

scaliness of paws and tail. Their skin appeared very loose owing to loss of subcutaneous fat. One rat from this group developed diarrhœa and died in the 11th week.

Histological observations.—Histological study revealed the following changes in the skin.

1. *Epidermis.*—Out of the 6 rats, 4 showed hyperkeratinization of the stratum corneum. In one case the hyperkeratinization was severe, whereas in other cases it was moderate. All the other layers appeared normal.

2. *Corium.*—Normal.

3. *Sebaceous glands.*—Normal.

4. *Hair follicles.*—Dilatation and plugging of the funnels of hair follicles by dense keratinous masses was observed in 4 cases. In the remaining 2 cases, they appeared normal.

There was loss of subcutaneous fat store in all the rats except one (Fig. 8).

The above results show that a fat-free diet, or a diet low in essential fatty acids gives rise to skin lesions which are microscopically very much similar to those found in vitamin A deficiency. A diet containing 5% hydrogenated oil which is low in essential fatty acids, was found to give rise to mild cutaneous lesions like slight hyperkeratinization, dilatation and plugging of hair follicles. This has been attributed to lack of essential fatty acids in the fat, since these are the only fatty acids which the animal body is found incapable of synthesising.

It was originally proposed to supplement the diet containing hydrogenated oil with pure linoleic acid and study the effect but since it was not available locally, gingelly oil was incorporated in the diet replacing part of the hydrogenated oil so as to give the concentration of about 1 gm. of linoleic acid in 100 gm. of the diet (*i.e.*, 2·5% vanaspati and 2·5% gingelly oil). When the animals were kept on this diet, their average daily intake of linoleic acid was about 63 mg. per rat per day, which is above the minimum curative dose recommended by several workers.

RESULTS

All the animals remained healthy throughout the period of experiment and histopathological examination of their skin did not reveal any abnormality.

The results of the experiment show that skin lesions like slight hyperkeratinization, dilatation and plugging of hair follicles observed in rats kept

on a diet containing 5% hydrogenated oil can be completely prevented by replacing a part of hydrogenated oil by gingelly oil, which contains 40% linoleic acid. An indirect proof is thus obtained that linoleic acid plays an important role in the maintenance of healthy skin.

Discussion

These comparative studies show that keratotic plugs can readily be induced in rats' skin when the animals are maintained on vitamin A deficient diets, or when kept on suboptimal levels of vitamin A. The cutaneous lesions produced were found to be identical in many respects with those described by Sullivan and Evans (1943) and Moult (1943) in their studies. These studies reveal the fact that the complicating deficiencies of the vitamin B complex, fat and essential fatty acids in experimental diets had been responsible for the varied interpretation of the vitamin A deficiency syndrome in the past.

Experiments on essential fatty acids also, stress the importance of the quality of fat in the diet on the production of cutaneous lesions in the rats. In 1929, Burr and Burr first described fat deficiency disease in rats and further showed that only the unsaturated fatty acids, linoleic, linolenic and arachidonic were responsible for the curative effects exerted by different fats. Since then several workers especially Graham *et al.* (1931), Turpeinen (1938), Hume *et al.* (1938) have observed typical skin symptoms of essential fatty acid deficiency in young rats. Barki *et al.* (1947) have shown that when adult rats are kept on a restricted caloric diet until they had lost one-half their weight and then placed on a fat-free diet *ad libitum*, they readily showed essential fatty acid deficiency symptoms, which were not observed when the animals were kept on the *ad libitum* regime right from the beginning. Decker *et al.* (1950) are of the opinion that mature mice on fat deficient diet fed *ad libitum*, though appear to be in a good condition remain in a chronic deficiency state. They were able to bring these mice to an acute deficiency state by imposing stresses like injuries, pregnancy or *x*-irradiation, proving that in chronic deficiency states, the animals were not able to withstand any stress. In this experiment the animals were kept on fat-free diet *ad libitum* for the initial 12 weeks and then onwards on restricted caloric diet.

The results obtained are in some respects similar to those reported by Ramlingswami and Sinclair (1953). Histological changes occurring in vitamin A deficiency are in complete agreement with the present results but in case of essential fatty acid deficiency, though hyperkeratinization and plugging of hair follicles with dense keratinous masses was prominent, acanthosis of the lining epithelium of hair follicles was not observed in any case. These

authors are of the opinion that essential fatty acids may be the cause of ' phrynoderma ' in man. However, the importance of vitamin A cannot be excluded, since the main characteristic features of ' phrynoderma ' were present in both types of deficiencies. A survey of the literature on ' phrynoderma ' brings forth the fact that, keratin is not always present in dense masses but may be in loose meshes and acanthosis of prickle cell layer is not a marked feature (Radhakrishna Rao, 1937 *a* and *b*). The patients suffering from ' phrynoderma ' are usually on a poor diet and lack of other factors may also be playing part in the causation of such changes as acanthosis, widened interpapillary processes, etc. Besides, experimentally it has been proved that acanthosis of prickle cell layer is characteristic of pyridoxine, pantothenic acid and biotin deficiencies (Follis, 1948).

The knowledge gained from such experiments have a practical application in the treatment of ' phrynoderma '. Studies, on serum fatty acids in the cases of ' phrynoderma ' by Menon *et al.* (1950) indicate that iodine number of serum fatty acids were significantly lowered. Cure was brought about by gingelly oil, which is a rich source of essential fatty acids. Gopalan (1947) treated cases of ' phrynoderma ' by combined treatment of linseed oil and yeast extract. The author felt, that the failure of vitamin A concentrate to appreciably influence the skin condition in cases of ' phrynoderma ' and the success obtained with raw linseed oil would suggest that the curative effect claimed for the fish liver oils is probably due to their essential fatty acids content.

As far as the experimental evidence goes [Burr and Burr (1930), Salmon (1938), Hume *et al.* (1940)] it is to be noted that fish liver oils were not found very effective in curing the fat deficiency dermatitis in rats. Besides Frazier and Hu (1931, 1935, 1936), Lowenthal (1933), Reiss (1936), Youmans (1937) and Goodwin (1934) have all reported the disappearance of the lesions following the administration of cod-liver oil. Radhakrishna Rao (1938) cured two advanced cases of ' phrynoderma ' by administration of vitamin A concentrate. Finally, the animal experiments carried out by Moult (1943) as well as the results of the present experiment show that vitamin A is required for the maintenance of healthy skin. In its absence, lesions were produced which were found homologous to those occurring in cases of ' phrynoderma '. Rats used in the present experiment were on a diet containing peanut oil at 5% level which on the basis of average food consumption provided 130 mg. of essential fatty acids. This dose of essential fatty acids which is much above the minimum curative dose required for curing fat deficiency disease in rats, was found incapable of preventing the skin lesions in absence of vitamin A. On this point, our results tend to lend support to Radhakrishna

Rao's (1937) hypothesis that ' phrynoderma ' is a manifestation of nutritional deficiency, in which lack of vitamin A is an important factor.

It is worth mentioning here that all Gopalan's patients showed frank signs of vitamin B deficiency and the beneficial effect of yeast in all likelihood may be due to its indirect favourable influence on the utilization of essential fatty acids. The validity of this view is borne out by the finding of Menon *et al.* (1950) who have succeeded in curing ' phrynoderma ' with gingelly oil alone without the use of vitamin B supplements. Thus it is evident that clinical cases of ' phrynoderma ' (uncomplicated by other vitamin B complex deficiencies) can be cured by the administration of essential fatty acids alone, and the influence of B vitamins in this connection is only indirect. The fact that skin lesions in experimental animals paralleling ' phrynoderma ' can be produced even on yeast diets in the absence of essential fatty acids, gives further proof that the role of B complex deficiencies in the actiology of ' phrynoderma ' is secondary.

The results of the present experiment tend to show that both vitamin A and essential fatty acids are absolutely necessary in the diet and the lack of either factor gives rise to lesions which are microscopically homologous to those occurring in ' phrynoderma '.

Summary and Conclusions

This investigation was undertaken to elucidate the actiology of ' phrynoderma'. Two experiments were conducted, one to study the effect of vitamin A deficiency and the other, to study the part played by fat in the production of cutaneous lesions in rats.

It is concluded that deficiency of vitamin A in conjunction with deficiency of essential fatty acids, plays an important role in the production of cutaneous lesions in rats resembling those in ' phrynoderma '. It is suggested that ' phrynoderma ' may be due to the combined deficiencies of both these factors. The experimental evidence further points out, that lack of vitamin A plays a larger role in the production of cutaneous lesions resembling ' phrynoderma'.

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FIG. 1. Rat on a completely vitamin A deficient diet, showing typical xerophthalmia of both the eyes.

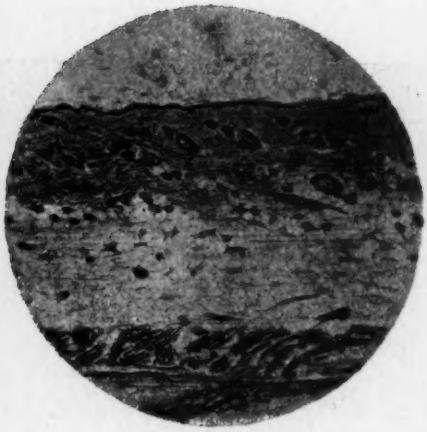

FIG. 2. Skin section of a rat receiving 50 I.U. vitamin A per day, apparently in normal health (control), ×50.

FIG. 3. Skin section of a rat receiving 1 I.U. vitamin A per rat per day, showing moderate amount of hyperkeratinization of stratum corneum layer, with dilatation and plugging of hair follicles with keratinous material. Fair amount of subcutaneous fat is present, ×50.

FIG. 4. Skin section of a rat on a completely vitamin A deficient diet, showing marked hyperkeratinization of stratum corneum layer, with dilatation and plugging of hair follicles with keratinous material and loss of subcutaneous fat, ×100.

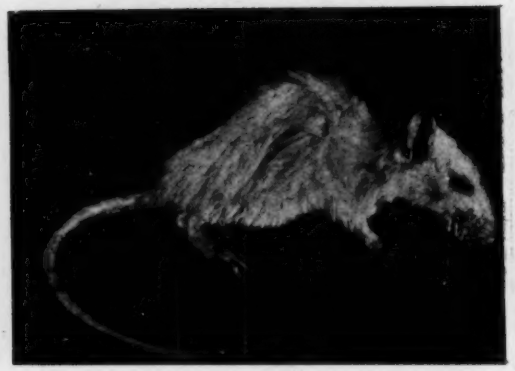

FIG. 5. Rat on a completely fat-free diet, showing scaly dermatitis of the paws, tail and pinnæ of the ear, dishevelled fur and marked emaciation. Eyes are normal.

FIG. 6. Skin section of a rat on a diet containing 5% hydrogenated oil, showing moderate amount of hyperkeratinization of stratum corneum layer, with dilatation and plugging of some hair follicles with keratinous material and loss of subcutaneous fat, × 50.

FIG. 7. Skin section of a rat on a fat-free diet containing 70% sucrose, showing marked hyperkeratinization of stratum corneum layer, with marked dilatation and plugging of hair follicles with corneum layer, with keratinous material and loss of subcutaneous fat, ×100.

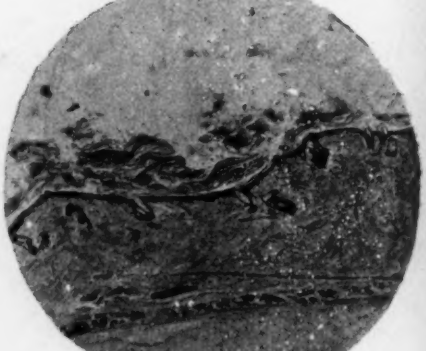

FIG. 8. Skin section of a rat on a fat-free diet, containing 50% starch and 20% sucrose, showing marked hyperkeratinization of stratum corneum layer, with slight dilatation and plugging of few hair follicles and loss of subcutaneous fat, ×50.

Figs. 2–4, 6–8. Ehrlich's acid hæmatoxylin and eosin stain.

THE EFFECT OF TRACE ELEMENTS ON NITROGEN FIXATION BY *AZOTOBACTER*

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Introduction

Trace elements have long been known to affect the process of nitrogen fixation by *Azotobacter*. Remy and Rosing (1911) observed that Fe stimulated nitrogen fixation while Baier (1936) found that improvement of growth and activity of the organism by sewage and humic acid was mostly due to iron in them. Kayser (1921) noted that U-salts enhanced nitrogen fixation. The importance of Mo in the process is now well known (Bortels, 1930, 1936; Burk, 1934; Nilsson, 1936; Burk and Horner, 1940; Burema and Wieringa, 1942; Mulder, 1948). Schröder (1932) considered Cu and Zn to be necessary for the growth of *Azotobacter* while Mulder (1939) found that *Azotobacter chroococcum* needed Cu for the production of dark pigment. Von Linden (1934) observed that Cu facilitated oxidative processes and stimulated growth through its action on the cell plasms. Burk and Horner (*loc. cit.*) observed that Va could partly replace Mo in its effect on nitrogen fixation. Plaut and Lardy (1949) noted that the oxalacetate decarboxylase of *Azotobacter* was activated better by Mn than by Mg. Jordan and Anderson (1950) observed increased nitrogen fixation in soils treated with B when the availability of B ranged from 0·5 to 1·8 p.p.m.

In the present investigation, the effect of trace elements Mo, Va, U, Cu, Mn, Fe^{++}, Fe^{+++}, Co, Ni and Pb on nitrogen fixation by *Azotobacter* in Fred and Waksman's medium (1928) was tried. The effect of some of these cations on nitrogen fixation by *Azotobacter* was also studied in Jensen's medium (1951) containing sucrose where decreases or increases in nitrogen fixation are usually much more than in the former.

Experimental

The medium No. 77 due to Fred and Waksman (*loc. cit.*) was used in some of the determinations.

Analytically pure salts of the following were recrystallised and added to the medium in the following concentrations: 0·02, 0·1 and 1·0 p.p.m. Mo (Sodium molybdate), Va (Sodium vanadate), U (Uranium acetate), Cu (Copper sulphate), Mn (Manganese sulphate), Fe^{++} (Ferrous sulphate). The following trace elements were added in concentrations 0·02, 0·1, 1·0, 2·0, 3·0, 4·0, 5·0 and 10·0 p.p.m. Fe^{+++} (Ferric sulphate), Co (Cobalt sulphate), Ni (Nickel sulphate), Pb (Lead acetate).

The following combinations in concentrations of each constituent giving individually the maximum fixation of nitrogen was tried in the medium (1) Co *plus* Ni, (2) Ni *plus* Fe, (3) Fe *plus* Co and (4) Fe *plus* Co *plus* Ni.

Jensen's medium (Jensen, 1951) containing sucrose was used in which Fe, Co and Ni were substituted in place of Mo at the same concentration. The effect of such replacements on nitrogen fixation by *Azotobacter* was studied. The effects of these elements together with Mo on nitrogen fixation by *Azotobacter* were also studied.

RESULTS

The amounts of nitrogen fixed when trace elements were added are given in Table I.

TABLE I

Effect of trace elements on fixation of nitrogen by Azotobacter

(Figures of nitrogen fixed are in mg. per gm. of mannite, concentrations used are in parts per million)

Element	Concentration	Nitrogen fixation		Growth
		Average	Range	
No treatment ..	..	6·06	(6·0—6·7)	Appreciable growth, yellow pigmentation
Molybdenum ..	0·02	8·20	(8·0—8·5)	Good growth in all cases
„ ..	0·10	8·13	(7·8—8·5)	do.
„ ..	1·00	6·47	(5·9—7·0)	do.
Vanadium ..	0·02	6·20	(6·1—6·3)	Good growth slight
„ ..	1·00	6·07	(5·9—6·2)	do. pigmentation in all cases
„ ..	0·10	7·10	(7·0—7·2)	do.

TABLE I (*Contd.*)

Element	Concentration	Nitrogen fixation		Growth
		Average	Range	
Uranium ..	0·02	6·20	(6·0—6·5)	Heavy scum in some case, almost dark, solution turbid
„ ..	0·10	6·13	(6·0—6·3)	Moderate scum, coloured brown, solution turbid
„ ..	1·00	7·27	(7·2—7·3)	Slight brown-coloured pellicle
Copper ..	0·02	5·60	(5·4—5·8)	Fair growth, faintly brown to light pellicle
„ ..	0·10	5·47	(5·4—5·6)	Good growth, faint brown-coloured pellicle, solution turbid
„ ..	1·00	6·00	(6·0—6·0)	Copious growth *deep* chocolate-coloured pellicle, solution brown
Manganese ..	0·02	7·43	(7·2—7·7)	Fair growth, pink-coloured solution
„ ..	0·10	7·17	(7·0—7·3)	Profuse growth with pink-coloured solution
Iron (ous) ..	0·02	6·13	(6·0—6·3)	Appreciable surface growth of definite pink colour
„ ..	0·10	6·33	(6·3—6·4)	Less surface growth, colour of the pellicle slightly pink

TABLE I (*Contd.*)

Element	Concentration	Nitrogen fixation		Growth
		Average	Range	
Iron (ic) ..	0·02	7·63	(7·5—7·7)	Appreciable surface growth of slightly yellow colour
„ ..	0·10	7·63	(7·5—7·7)	Good growth, colour of the pellicle light yellow
„ ..	1·00	8·27	(8·1—8·5)	Good surface growth, colour of the pellicle slightly brownish
„ ..	2·00	8·20	(8·1—8·3)	Fair growth in all the cases. Pigmentation not noticeab e
„ ..	3·00	8·20	(8·1—8·3)	do.
„ ..	4·00	8·10	(8·0—8·2)	do.
„ ..	5·00	8·10	(8·0—8·2)	Appreciable growth in all the cases, solution slightly brown
„ ..	10·00	7·83	(7·8—8·7)	Good growth, no pigmentation except in one
Cobalt ..	0·02	8·70	(8·6—8·8)	Heavy growth, pigmentation on the sides of the pellicle in all cases
„ ..	0·10	8·47	(8·2—8·6)	do.
„ ..	1·00	8·43	(8·2—8·6)	do.
„ ..	2·00	8·27	(8·1—8·5)	do.
„ ..	3·00	8·27	(8·2—8·3)	do.
„ ..	4·00	8·20	(8·1—8·3)	do.
„ ..	5·00	8·17	(8·0—8·3)	do.
„ ..	10·00	7·83	(7·8—8·7)	do.

TABLE I (*Contd.*)

Element	Concentration	Nitrogen fixation		Growth
		Average	Range	
Nickel ..	0·02	6·00	(5·9—6·7)	Good growth with brown pigmentation
,, ..	0·01	7·96	(7·9—8·1)	Heavy growth with no pigmentation
,, ..	1·00	8·70	(8·6—8·8)	Fair growth with no pigmentation
,, ..	2·00	8·43	(8·4—8·5)	Fair growth with no pigmentation
,, ..	3·00	8·27	(8·2—8·3)	Fair growth with no pigmentation
,, ..	4·00	..	..	No growth
,, ..	5·00	..	..	No growth
,, ..	10·00	..	..	No growth
Lead ..	0·02	6·13	(5·9—6·5)	Growth in all cases
,, ..	0·10	6·00	(5·8—6·3)	do.
,, ..	1·00	5·63	(5·3—6·3)	do.
,, ..	2·00	5·77	(5·5—6·0)	do.
,, ..	3·00	5·53	(5·3—5·7)	do.
,, ..	4·00	5·57	(5·4—5·8)	do.
,, ..	5·00	5·27	(5·1—5·4)	do.
,, ..	10·00	4·73	(4·5—5·1)	do.

From Table I it can be seen that Mo, Mn, W increased nitrogen fixation at a concentration of 0·02, 0·1 and 1·0 p.p.m. while Fe^{+++} and Co increased nitrogen fixation at these concentrations and also at higher concentrations of 5·0 and 10·0 p.p.m. It may also be mentioned that hitherto the beneficial effects of Co on nitrogen fixation by *Azotobacter* do not seem to have been recorded. In view of the fact that vitamin B_{12} contains Co and this vitamin is known to be synthesised by *Azotobacter*, this effect of Co on nitrogen fixation seems to have some special significance and needs further investigation. Fe^{+++} and Ni at concentration of 0·1, and 1·0 p.p.m. enhanced nitrogen fixation while U was effective at a concentration of 1·0 p.p.m. and Va at a

concentration of 0·1 p.p.m. Beyond 3·0 p.p.m. Ni was toxic. Nitrogen fixation remained unaffected in presence of Pb in concentrations up to 0·1 p.p.m. With higher additions of Pb the nitrogen fixation gradually fell. The relation between concentration of Pb in the medium and the amount of nitrogen fixed by *Azotobacter* in such media could be expressed in the form of the equation

$$y = 5{\cdot}5884 - 0{\cdot}4035 \ \log_{10} x$$

where x is the concentration of Pb in parts per million in the medium and y, the amount of nitrogen fixed in mgm. per gm. of mannite.

Noticeable differences could be observed in the effect of these trace elements on the pigment formation by *Azotobacter* in culture solution.

Table II shows the amounts of nitrogen fixed when different trace elements were added in combinations at concentrations where they individually gave maximum nitrogen fixation.

TABLE II

Fixation of nitrogen by Azotobacter *in presence of combinations of trace elements*

(Figures of nitrogen fixed are in mg. per gm. of mannite)

Combination Figures within brackets are concentration in p.p.m.	Nitrogen fixed (average of 3 replications)
Control (without addition of any trace element) (Original medium) ..	5·67
Co (0·02), Ni (1·0) ..	6·47
Ni (1·0), Fe (ic) (1·0) ..	7·20
Fe (ic) (1·0), Co (0·02) ..	7·17
Fe (ic) (1·0), Co (0·02), Ni (1·0) ..	6·60
C.D. at 1 per cent. ..	0·25

It is seen in Table II that Co and Fe and Ni *plus* Fe gave the maximum fixation of nitrogen and the effect of these combinations did not differ significantly from each other. Since Co and Ni are interchangeable with Fe to give a combination of trace elements yielding maximum fixation, it is likely

that Co, Ni and Fe are present at selected centres of activity on the cell surface and take part in some form in nitrogen fixation.

In order to study if any antagonistic or complementary effects could be had by combinations of the elements, the combination of several trace elements was studied using the relevant salts mentioned earlier. The results are given in Table III.

TABLE III

Effect of combinations of trace elements in presence of molybdenum on nitrogen fixation by Azotobacter

Combination of trace elements	Nitrogen fixed at concentration of trace elements giving individually the maximum fixation of nitrogen	
	Fred's medium (mg./gm. of mannite)	Jensen's medium (mg./gm. of sucrose)
Control without any trace element ..	5·70	4·40
Molybdenum ..	8·13	13·43
Molybdenum and Cobalt ..	6·63	11·53
Molybdenum and Nickel ..	6·45	12·10
Molybdenum and Iron (ic) ..	8·10	12·47
Molybdenum, Cobalt and nickel ..	6·47	11·98
Molybdenum, Nickel and Iron (ic) ..	6·50	11·75
Molybdenum, Cobalt and Iron (ic) ..	6·30	12·58
Molybdenum, Cobalt, Nickel and Iron (ic)	6·00	12·45
C.D. at 5 per cent. ..	0·33	0·30

From the data in Table III it could be clearly seen that except Fe the trace elements added in the given concentrations exerted an antagonistic effect against Mo.

In Table IV are given the nitrogen fixation in Jensen's medium when Mo was in concentrations used in the medium and when the element was

replaced by Co, Fe and Ni using the same concentrations as that of Mo in the form of sodium molybdate.

TABLE IV

Nitrogen fixation in Jensen's medium with Molybdenum and without Molybdenum but with Cobalt, Nickel and Iron

(Figures of nitrogen in mg. per gm. of sucrose oxidised)

Trace element	Nitrogen fixation (average of 3 replications)
No Molybdenum ..	4·37
Molybdenum ..	13·88
Cobalt ..	7·97
Nickel ..	6·62
Iron (ic) ..	6·62
C.D. 1 per cent. ..	0·16

It could be seen from the data in Table IV that highest fixation of nitrogen was observed in the case where Mo had been present and that Co, Fe or Ni could not replace Mo effectively to give the same amount of nitrogen fixation. It is also seen that the amount of nitrogen fixed by *Azotobacter* in the Fred and Waksman's medium was very much lower than those determined in Jensen's.

In Table V are given the effect of different concentrations of trace elements like Mo, Fe, Co, Ni on fixation of nitrogen in Jensen's medium. For the purpose of this study Mo was omitted in the medium when other trace elements were used.

It could be seen from the data in Table V that in Jensen's medium the concentration of Mo of 2·0 p.p.m. gave the highest fixation of nitrogen, and in the cases of Co, Ni and Fe the concentrations for highest nitrogen fixation are 0·1, 2·0 and 2·0 p.p.m. respectively. It was interesting to note that in Jensen's medium, nitrogen fixations remained unimpaired with higher concentrations of Mo, while the same concentrations showed remarkable decreases in fixation in the case of Co, Ni and Fe. Ni and Fe^{+++} at concentration of 5·0 p.p.m. stopped the growth of the organism altogether. It was thus clear,

TABLE V

Effect of different concentrations of Molybdenum, Iron, Cobalt and Nickel on nitrogen fixation by Azotobacter *in Jensen's medium*

(Figures of nitrogen fixed are in mg. per gm. of sucrose and are averages of three determinations)

Concentration of trace elements in p.p.m.	Molybdenum	Cobalt	Nickel	Iron	C.D. at 5%
Control without any trace element	4·37	4·37	4·37	4·37	Differences not significant
0·02	6·47	6·22	6·38	6·07	do.
0·10	6·35	9·00	6·23	5·88	0·27
1·00	13·10	8·35	3·22	8·22	0·16
2·00	13·82	8·22	8·38	8·23	0·39
3·00	13·25	8·20	8·00	7·70	0·33
4·00	13·25	7·90	6·95	7·78	0·16
5·00	13·88	7·95	0·00	0·00	0·26

that though these trace elements like Co, Ni and Fe caused significant increases in nitrogen fixations, they could not replace Mo effectively in Jensen's medium, at a concentration of 1·00 p.p.m.

In Table VI are given the effect of concentration of sugar on nitrogen fixation in presence of trace elements present in the medium in same concentrations in which Mo was present in original Jensen's medium.

From the data in Table VI it could be observed that higher fixations of nitrogen took place with and without the presence of trace elements, in the case where smaller amounts of sugar had been used. There was, however, a remarkable exception in the case of Ni. When smaller quantity of sugar was used for growth of *Azotobacter*, the organism was unable to grow and fix nitrogen, at a concentration of Ni which, however, allowed growth and nitrogen fixation when larger quantity of sugar was present. And at this concentration of Ni, the amount of nitrogen fixed was higher than the control. It was also seen from the data that whatever may be the quantity of energy material Mo gave the highest nitrogen fixation.

TABLE VI

Effect of concentration of sugar in the medium in presence of trace elements on nitrogen fixation by Azotobacter

(Figures of nitrogen fixed are in mg. per gm. of sugar)

Trace element	Nitrogen fixed (average of 3 replications)	
	10 gm. of sugar	20 gm. of sugar
Control No trace element ..	6·40	4·37
Molybdenum ..	16·46	13·90
Cobalt ..	8·17	7·97
Nickel ..	0·00	6·62
Iron (ic) ..	8·10	6·62
C.D. at 1 per cent. ..	0·15	0·16

Cell composition with respect to Co, Mo and Ni.—Since evidence of positive stimulation of nitrogen fixation was obtained with the use of the trace elements, Mo, Co and Ni, the cells of *Azotobacter* grown in media containing these elements were examined for the same. Mo was determined by Ferric Thiocyanate method (Piper, 1950), Co by the Nitroso-R-Salt method (Marston and Dewey, 1940) and Ni by the Dimethyl Glyoxime method (Sandell, 1944).

Both the media were tested for the presence of Mo, Co and Ni by evaporating 200 c.c. of each to 10 c.c. on water-bath, and testing for these elements. It was found that both the media contained traces of Co (about 0·00025 p.p.m.).

Azotobacter was grown in Jensen's medium containing the trace elements Co, Ni and Mo in concentrations equivalent to Mo that was usually present. After 14 days' growth, the *Azotobacter* cells were separated by drawing the pellicle from the surface of the culture liquids and then transferring them to large volumes of double distilled water. The suspensions were centrifuged and the cell sediments given several centrifugal washings with double distilled water. The cells were then transferred to silica basins,

dried, ignited and from the ash extracts Co, Ni and Mo were estimated by the colorimetric methods. In Table VII are given the relative amounts of these substances in the culture solutions and in the body cells of *Azotobacter*.

It is evident from the data in Table VII that definite absorption of the trace elements, Co and Mo, had taken place in the body cells of *Azotobacter*. The amount of Ni in the body cells of *Azotobacter* was too small to be considered significant. To confirm the absorption of Mo which was absorbed

TABLE VII

Distribution of Cobalt, Nickel and Molybdenum in culture liquids and Azotobacter *cells*

(Trace elements are exprsed in μg.)

Trace element	Amount originally present in culture liquid	Amount present in *Azotobacter* cells	Absorption per cent.
Cobalt ..	40	1·50	3·75
Nickel ..	2,000	1·04	0·05
Molybdenum ..	6,000	124·00	2·06

in smaller quantities than Co, spectrographs were taken of the cell ashes obtained from the growth in Jensen's medium with and without Mo. There was no indication of Mo line in the photograph of cell obtained from the Mo-free medium while the cells from the Mo-treated medium showed Mo lines thus indicating the absorption of Mo by the cells (Fig. 1).

SUMMARY AND CONCLUSIONS

Mo, Co, Ni and Fe (ic) have been found to stimulate nitrogen fixation significantly when occurring in traces in a medium allowing growth of *Azotobacter*. Mo and Co gave maximum nitrogen fixation when present in concentration of 0·02 p.p.m. while the same occurred at concentrations of 1·0 p.p.m. in the case of Ni and Fe (ic). With increase in concentrations of these elements, there was depression in nitrogen fixation though the values of the latter were still significantly higher than that observed in the case of control (without any trace element). The decrease in nitrogen fixation with increase in concentration of Ni and Fe (ic) was however found to be irregular.

Increasing concentration of lead brought about a gradual and regular decrease in fixation of nitrogen.

Combinations of Co, Mo, Ni or Fe (ic) gave lower fixations of nitrogen than that obtained by the use of Mo alone both in the Fred and Waksman's medium and in that of Jensen.

Highest fixation of nitrogen was observed in the Fred and Waksman's medium at a concentration of Mo at 0·02 p.p.m. The highest fixation of nitrogen in Jensen's medium was obtained at a concentration of 2·0 p.p.m. of Mo.

Nitrogen fixations in Jensen's medium were always less when Mo was replaced by Co, Ni or Fe (ic) than when Mo was used at 1 p.p.m. Mo when used in combination with any or other of these trace elements gave lower fixations of nitrogen in Jensen's medium.

Increase in concentrations of Mo in Jensen's medium brought about increase in fixation of nitrogen while the same in the other trace elements brought about depression in nitrogen fixation specially at higher concentrations.

Increase in amounts of energy material lessened the toxicity of Ni at higher concentrations as reflected in the fixations of nitrogen.

There was definite evidence of absorption of Mo and Co in the body cells of *Azotobacter*.

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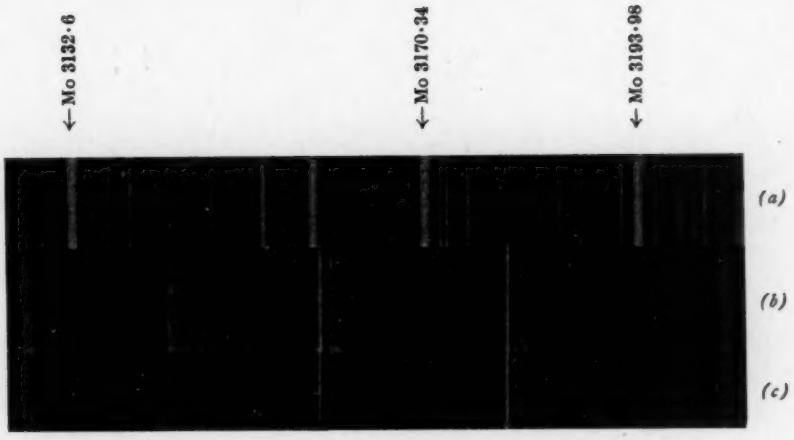

FIG. 1.

Taken with Hilger's Large Quartz Spectrograph

(*a*) Sodium molybdate L.R.
(*b*) Cell treated with molybdenum showing molybdenum line indicated by arrows.
(*c*) Control showing absence of molybdenum lines.

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DEVELOPMENTAL STUDIES

V. Di-, Tri- and Tetrachotomous Branching System in *Carissa carandas* Willd.

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The branching system of *Carissa carandas* (Apocynaceæ) has been described in books on taxonomy as *dichotomous*—a phenomenon rare in dicotyledons. The two branches at each forking are unequal and they stand nearly at right angles to each other, the major ones apparently continuing the forward extension of the primary axis. As a result the primary axis appears zigzag and sympodial in construction. The leaves are opposite. The first pair of leaves borne on the major branch are not associated with the thorns, but they bear buds in their axils which remain dormant unless forced into development. The next pair borne just below the forks " are crossed with the thorns". The thorns occur in pairs at each forking, are opposite, horizontal and frequently fork in their turn. The branches (daughter axes) are borne in the axils of the leaves and the thorns, in the axils of the scale leaves (Roxburgh, 1875; Hooker, 1882) (Fig. 1).

Neither Roxburgh, nor Hooker, nor any later taxonomists appear to have studied the origin and nature of the forks (branches) in development, their vascularization and relationship with the thorns. The present report embodies the results of such studies of these structures at the shoot apex of *Carissa carandas*.

Materials for the study were collected from plants growing in the garden of the University of Calcutta. Usual methods of fixation, dehydration, etc., have been followed in the preparation of paraffin blocks. Microtome sections, both transverse and longitudinal, were cut at 8–10 μ, stained in safranin and fast-green combination and made permanent. Figures are all Camera lucida drawings.

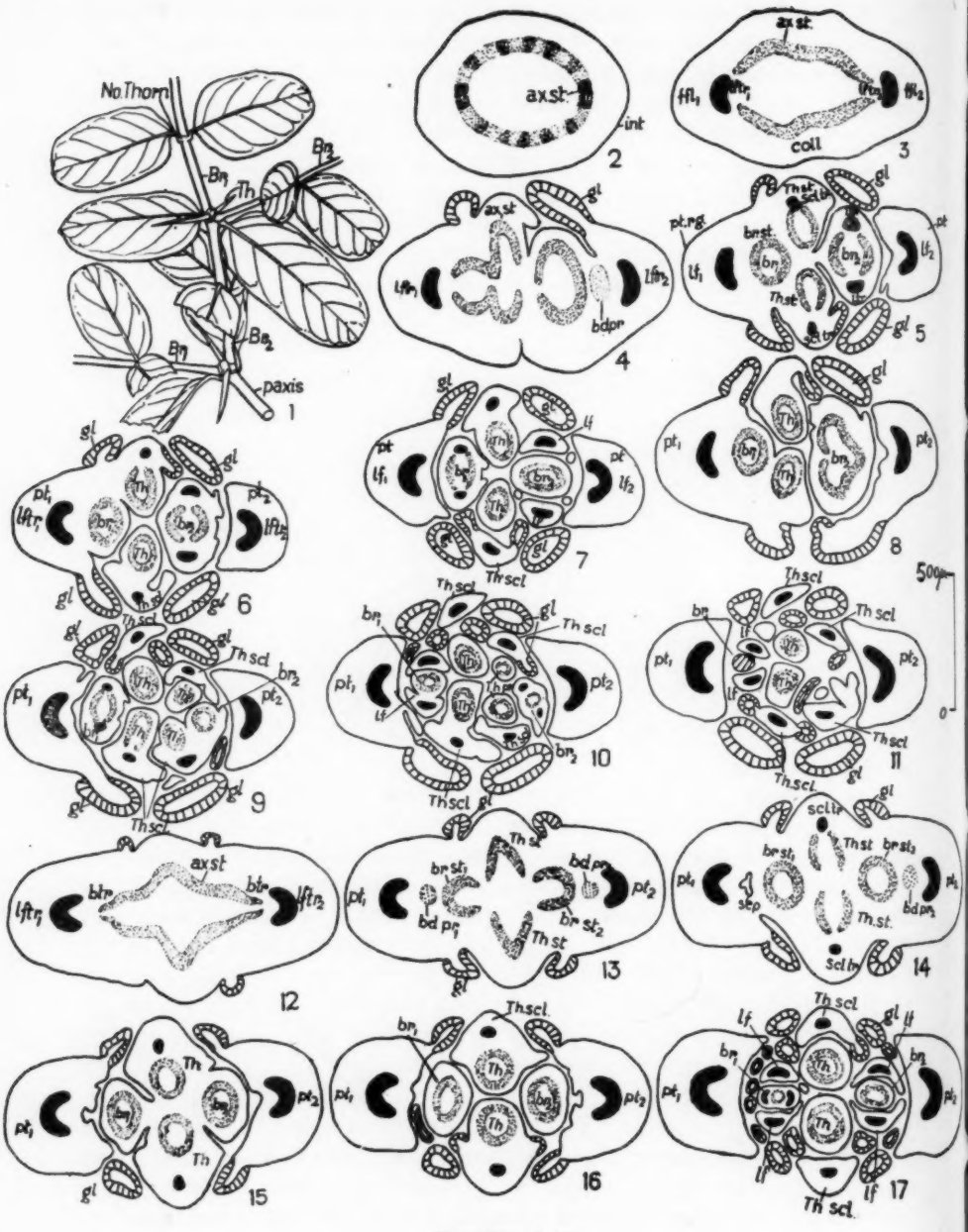
1. *Dichotomous branching of the parent axis, and trichotomy of one of the daughter axes to give rise to the second branch and a pair of thorns* (Figs. 2–7).

Figure 2 shows the structure of a very young internode in transverse section just below the node. The central stele consists of a ring of provascular meristem in which the vascular strands are in the process of differentiation. In Fig. 3 which is a transverse section at the base of the node a *collar* (*coll.*) has formed round the axis by the fusion of the extended arms of the foliar foundations (ffl_1 and ffl_2) of the pair of opposite leaves; two procambial strands, leaf traces of the pair of opposite leaves (lf_1 and lf_2), have just left the central stele causing two gaps (leaf gaps) in the latter which now shows a wavy contour with two lateral folds which stand opposite to and face each other. In Fig. 4 the leaf trace bundles ($lftr_1$ and $lftr_2$) have moved further in the foliar foundations. Glands* have appeared at the margins of the petiolar regions of the foliar foundations, and primordium of axillary bud* in the axil of $leaf_2$, has also been organized opposite the leaf trace, but it soon disappears.

In the next figure (Fig. 5) bifurcation of the parent axis is almost complete, and of the two daughter axes—the right-hand one directly develops into a major branch and the other divides into three parts: the central and two lateral ones. The central one develops into the second branch of the fork, and the lateral ones, into a pair of thorns. The branches stand in the axils of the pair of leaves and the thorns in those of scale leaves (*see* Fig. 7). Though the two branches and the pair of thorns are associated with the axillant leaves and scales, they are not produced from axillary buds but in the first instance by the bifurcation of the parent axis and then by the trichotomy of one of the daughter axes. There is no terminal bud to prolong the growth of the parent axis.

Vascular adjustments precede bifurcation of each parent axis, and the trichotomy of a daughter axis. The parent stele divides into two unequal parts—the right hand portion forms the stele of the major branch (br_2) and the left hand portion shows three parts—a central one and two lateral V-shaped ones (Fig. 4). The three parts then separate from one another to constitute steles of the branch (br_1) and the two thorns (*Th*). From the thorn-steles two trace bundles, one for each scale leaf—are given out to the still attached scales which ultimately separate from their respective thorns. Glands have now formed on the margins of the thorn scales (Figs. 4–6). In Fig. 6 daughter axis (br_2) has developed foliar foundations of a pair of opposite leaves with their traces. The left-hand daughter axis now organizes and separates into three parts: a pair of thorns (Th_1) and (Th_2) with scales and

* The origin and nature of the glands and axillary buds have been omitted from this report.



TEXT-FIGS. 1–17

the foliar foundation of leaf$_1$ still attached to the central part which will develop into the left-hand branch (br_1). The steles of the thorns and the axis (br_1) have been fully organized.

In Fig. 7 the right-hand daughter axis has developed into an embryonic shoot with a pair of opposite leaf primordia; the axillant leaf has separated from it. The central portion of the left-hand daughter axis has organized into a branch with leaf traces just separated from the axial stele and the two lateral divisions have developed into a pair of thorns one with the scale separated and the other with the axillant scale still attached to it.

2. *Dichotomy of the parent axis and trichotomy of both the daughter axes* **(Figs. 2, 3, 8–11).**

Figures 2 and 3, as in the previous case, depict the first stages leading towards the division of the parent axis. Figure 8 shows the right-hand daughter axis with the stele differently contoured (*cp.*, Fig. 4). Figure 9 shows a similar trichotomy of this axis as described for the left-hand daughter axis in the previous case (*cp.*, Figs. 5–7). Figure 10 shows the primordia of the branch (br_2) and the two thorns—products of the trichotomy of the daughter axis. These primordia with the scales of the pair of thorns are, however, feebly developed. Figure 11 shows these primordia—instead of developing into a branch and a pair of thorns, become atrophied and then disappear.

In this case the axis at the node shows a pair of opposite leaves, one branch and a pair of thorns (developed from the left-hand daughter axis).

3. *Tetrachotomy of the parent axis leading to the formation of a pair each of the branches and the thorns* **(Figs. 12–17).**

Figure 12 shows the structure almost similar to that shown in Fig. 3, but the lateral folds of the axial stele is centrally disposed, *i.e.*, equidistant from the two leaf gaps. Traces of axillary buds are sent out from the free ends of the central cylinder flanking these gaps. Figure 13 shows the central cylinder broken up into 4 pieces, the left and right hand ones assuming closed rings, and the lateral infoldings becoming V-shaped. Two axillary bud primordia appear and glands begin to develop at the margins of the foliar foundations.

Figure 14 depicts the stage when the scale traces of the thorns are given out from the apices of V-shaped stele; the axillary bud primordium has disappeared from the axil of leaf$_1$; separation of the foliar foundations from the axis has just began opposite the bud primordium leaving a depression in the

foliar foundation indicating the foliar origin of the axillary bud which is ephemeral, more glands have developed.

Figure 15 shows tetrachotomy of the parent axis which has started from the outside with the four pieces of the central cylinder as their respective steles. Petioles of both the leaves have separated from the branches (daughter-axes), but the thorns are still attached to their respective scales. Figure 16 shows entire separation of the two branches and the two thorns from one another, but the thorns are still confluent with their axillant scales. Glands have also developed at the margins of the scales and also on the adaxial face of the petiole.

In Fig. 17 the daughter axes have started developing into a pair of branches, the first pair of leaves have already formed; the thorns have also developed in the axils of their respective axillant scales. More glands are seen in the section.

CONCLUSION

1. Branching system in this species is in the first instance dichotomous and then one of the daughter axes by undergoing trichotomy produces the second branch, and a pair of thorns at the bifurcation.

2. These branches are not produced by axillary buds, nor is there any terminal bud to continue the elongation of the primary axis which in this case is a pseudopodium, allied to, but not like, the scorpioid system of branching.

3. Before the parent axis divides foliar foundations of the pair of leaves with their trace bundles are well differentiated. They go with the two daughter axes. The branches when separated appear in the axils of these leaves, as do the normal axillary buds. From the thorn primordia also the scales separate with their trace bundles derived from the thorn-steles, and the thorn appear in their axils.

4. Axillary bud primordium organizes in the axil of $leaf_2$ and appear to be foliar in origin and are ephemeral, they are seen to fall off soon.

5. The nature of branching should not be confused with that of the cymose type. In the latter type the apical bud either dies off or a vestige remains. In the case of true dichotomy, which is practically confined to the lower plants, the apex divides into two daughter apices. This exactly happens in *Carissa carandas*. Moreover, trichotomy and tetrachotomy are also a common feature in this species.

6. The branching in this species is not monopodial, but sympodial. After bifurcation the thornless daughter axis develops into a major branch, the first pair of leaves produced on it are normally without branches and are thornless. At the next node forking takes place as described for the parent axis, but the major branch develops alternately right and left, and these major branches continue the forward growth of the parent axis.

Acknowledgement

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For description see text. (Forking, etc., have been studied in serial transverse sections of the node at the shoot apex.)

Explanation of Text-Figures

Text-Figs. 1–17. Fig. 1. A short twig of *Carissa carandas* showing the branching habit of the plant, ×½. Fig. 2. Transverse section of the internode at the base of the node. Fig. 3. Transverse section through the base of the node. Fig. 4. Transverse section of the node showing dichotomy of the parent axis. Figs. 5–7. Transverse sections of the shoot apex showing trichotomous branching of the left-hand daughter axis. Figs. 8–11. Transverse sections of the shoot apex. Figs. 8 and 9 show trichotomy of both the daughter axes. Figs. 10 and 11 show the atrophied nature of the right-hand thorns and branch. Fig. 12. Transverse section at the base of the node. Figs. 13–14. Transverse sections of the node. Figs. 15–17. Transverse sections of the shoot apex showing tetrachotomy of the parent axis.

axst, axial stele; *bdpr*$_1$, *bdpr*$_2$, bud primordia; *br*$_1$, *br*$_2$, forks (branches); *brst*, *brst*$_1$, *brst*$_2$, stele of branches; *btr*, bud trace; *coll*, collar; *ffl*$_1$, *ffl*$_2$, foliar foundations of the pair of leaves at the node; *gl*, glands; *int*, internode; *lf*$_1$, *lf*$_2$, leaves; *lf*, leaf on the branches; *lftr*$_1$, *lftr*$_2$, leaf trace bundles; *ltr*, trace bundle of the leaves on the branch; *paxis*, parent axis; *pt*, *pt*$_1$, *pt*$_2$, petiole; *scl*, scale leaves; *scltr*, trace of the scale leaves; *sep*, separation of the collar from the axis starts; *Th*, thorn; *Thpr*, thorn primordium; *Thscl*, scale leaves of the thorn and *Thst*, stele of the thorn.

A KEY TO THE GENERA OF THE COMMON GRASSES OF LUCKNOW AND ITS ENVIRONS

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Introduction

With a view to making a cyto-systematic study of the flora of Lucknow, a survey was made of the grasses around an area of one mile radius from the Central Drug Research Institute where the Central Botanical Laboratory was temporarily located during the period 1955–57. Collections were made also from Bangla Bazaar, a village not far from the Sugarcane Research Institute on the outskirts of the city, from Amausi Airfield about 8 miles to the south-west and from Chinhat Lake, 8 miles to the east of the city. An enumeration of grass species collected is given later along with additional data for each species regarding the locality of occurrence, the habitat and the date of collection in the flowering state.

A key is presented, in this paper, to the genera of grasses collected and now housed in the herbarium of the Central Botanical Laboratory, Allahabad. A detailed description of the species enumerated may be found in Blatter and McCann (1935) and Bor (1940, 1947).

Attention has to be directed to certain nomenclatural changes in the enumeration. In accordance with the alteration in the circumscription of the genus *Saccharum* by Dr. Jacob Jeswiet (*vide* Rosenfeld, 1956), *Saccharum munja* Roxb. has been transferred to *Erianthus* by him and becomes *Erianthus munja* Jeswiet (*cf.* Raizada, 1954). According to Reeder (1948), *Digitaria marginata* Link or *D. adscendens* Henr. becomes *D. sanguinalis* Scop. Reeder notes that this species is extremely variable, the spikelets ranging from glabrous to strongly fimbriate and that the species has a wide geographical range in both hemispheres. Reeder also gives a detailed synonymy for the species hitherto known as *Pseudoraphis aspera* Pilger and now correctly named *P. squarrosa* Chase.

In *Oryza*, the interpretation of the structure of the spikelet is still being debated. According to the commonly held view (presented in the generic

key below) the single flower of the spikelet is invested by the large lemma and its associated palea. Parodi (1946) prefers to regard the palea as a second lemma, the associated paleas of both the lemmas having disappeared and the flowers reduced to one in the spikelet.

Since the publication of the Gramineæ by Hooker (1897) some genera like *Panicum* and *Andropogon* have been split up and the circumscriptions of other genera altered by agrostologists so that now there are a large number of genera with relatively few species in them. It is hardly necessary to stress the need for generic keys for local floras based on current classification.

Key to the Genera

A Spikelet laterally compressed, the florets 2—many, generally several. Lemmas mostly exerted from involucral glumes. Rachilla usually disarticulating above the glumes and between the florets, rarely the spikelet falling entire or the rachilla persistent.

B Spikelets not crowded densely and unilaterally on the rachis. Lemmas 3-nerved, awnless.

C Spikelets pedicelled, in open or contracted panicles. Rachilla and lemmas glabrous or minutely hairy. Paleas 2-keeled .. *Eragrostis*

CC Spikelets very minute, sessile or shortly pedicelled, alternate and unilaterally 2-seriate on the very slender spiciform branches of the lax panicle. Paleas 2-nerved .. *Leptochloa*

BB Spikelets sessile, crowded densely and unilaterally on the rachis. Paleas 2-keeled.

C Spikelets on a very short rachis (about 2 cm.). Rachises arranged on a prolonged central axis, the whole inflorescence narrowly columnar or pyramidal. Glumes very unequal, lemmas 3-nerved, awnless. Spikelets falling entire. .. .. *Desmostachya*

CC Spikelets not falling entire, glumes sub-equal.

D Spikelet-bearing rachis terminating in a spikelet. Rachises in a terminal umbel (with sometimes one or more below) or

in whorls towards the apex of the culm .. .. .. *Eleusine*

DD Spikelet-bearing rachis terminating in a rigid point. Rachises 2–6, in terminal umbels .. .. *Dactyloctenium*

AA Spikelet awnless, with a single floret and two involucral glumes. Rachilla disarticulating above the two glumes.

B Spikelet pedicelled, very small, variously panicled. Glumes and lemma delicately membranous, the lower glume relatively small .. .. .. *Sporobolus*

BB Spikelet laterally compressed, sessile, imbricate, alternately 2-seriate and unilateral on a slender keeled rachis. Rachises 2–6 in terminal umbels. Glumes strongly keeled .. .. *Cynodon*

AAA Spikelet rounded or plano-convex, with two florets, the upper hermaphrodite or rarely female, the lower male or barren. Rachilla not produced beyond the upper floret. Spikelets falling entire at maturity. Lemma of fruiting floret papery to very tough, firmer than the glumes.

B Spikelets not in one-sided spikes or one-sided racemes.

C Spikelets without subtending bristles.

D Spikelets in more or less open panicles, the branches not prolonged beyond the uppermost spikelet. Lower glume often reduced .. .. *Panicum*

DD Spikelets in panicles, the branches produced as bristles beyond the uppermost spikelet. Lower glume minute, orbicular .. .. *Pseudoraphis*

CC Spikelets with subtending bristles. Glumes unequal.

D Spikelets in contracted spike-like or more or less open panicles. Bristles persisting after the fall of the spikelets .. .. *Setaria*

DD Spikelets 1–3 in involucels of bristles connate at the base, the inflorescence a

spike-like raceme. Bristles falling with the spikelet .. *Cenchrus*

BB Spikelets in one-sided spikes or one-sided racemes.

C Lower glume absent or rudimentary.

D Spikelets pedicelled on the spiciform branches of the raceme or panicle, the lowest internode of the rachilla (at the base of the spikelet) thickened and with cupular rim representing the lower glume .. .. *Eriochloa*

DD Base of the spikelet not so thickened, the spikelet awnless.

E Spikelets plano-convex, the pedicels very short and obscure, the flat side of the spikelet turned away from the rachis. Rachises 2-nate, digitate or spread out on the main axis. Palea much exposed between inrolled margins of the upper lemma .. *Paspalum*

EE Spikelets usually 2–3-nate and with unequal pedicels on the rachis. Rachises digitate or more or less distant on the main axis. Palea little exposed between overlapping margins of the upper lemma .. *Digitaria.*

CC Lower glume present, reduced.

D Lower glume generally turned towards the rachis. Spikelets awnless, distant or crowded, irregularly 1–2-(rarely 3-) seriate on the slender or spiciform branches of a simple raceme. Pedicels unequal if paired or very short. .. .. .. *Brachiaria*

DD Lower glume turned away from the rachis.

E Spikelets sub-sessile, close, 2- or irregularly pluriseriate on the abaxial side of the rachis. Rachises on a common axis. Upper lemma mucronate or short awned .. *Urochloa*

EE Spikelets awnless, sessile, glabrous, 2-seriate and close-set on the abaxial side of the flat rachis. Rachises alternate and rather distant on the main axis. Upper lemma acute. .. .. *Paspalidium*

EEE Spikelets often awned from lower lemma, more or less hispid, sub-sessile, in pairs or clusters on the abaxial side of the rachis. Rachises racemose on the main axis. .. *Echinochloa*

CCC Glumes sub-equal, lower or both awned. Spikelets sub-sessile, crowded or approximate, usually on the abaxial side of a rachis. Rachises alternate on the main axis. Prostrate or ascending grasses with lanceolate leaves .. *Oplismenus*

AAAA The spikelet typically with two florets, the upper hermaphrodite or female, the lower male or barren, but pedicelled spikelet sometimes with male floret only or barren or reduced to pedicel. Spikelets falling entire at maturity. Glumes as long as the spikelet and enclosing the florets, more or less rigid and firmer than the lemmas which are hyaline or membranous.

B Internodes of rachis and pedicels filiform.

C Spikelets awnless, 2-nate, one short, the other long pedicelled, disarticulating from the pedicels. Inflorescence a sub-cylindric silvery-hairy panicle .. .. *Imperata*

CC Spikelets in pairs, one pedicelled, the other sessile.

D Sessile and pedicelled spikelets alike and perfect, the pedicelled falling from pedicel, the sessile deciduous with contiguous joint of rachis and pedicel.

E Spikelets awnless, fertile lemma much reduced or wanting. Inflorescence a wide silvery-plumose terminal panicle .. .. *Saccharum*

EE Spikelets sometimes with a visible awn, fertile lemma with mid-nerve extending into a short mucro or prominent awn. Inflorescence a dense terminal silky panicle .. *Erianthus*

DD Pedicelled and sessile spikelets dissimilar, the sessile perfect, the pedicelled male or neuter.

E The sessile spikelet sometimes awned, the pedicelled awnless and sometimes reduced to glumes or glume or pedicel only. Inflorescence a spreading or compacted panicle of few-noded racemes, not silvery-plumose .. .. *Sorghum*

EE Sessile spikelet awned, the pedicelled awnless. Lower glume of sessile spikelet narrow, the joints and pedicels longitudinally grooved. Racemes shortly peduncled, usually subdigitate .. *Bothriochloa*

EEE Sessile spikelet generally awned, the pedicelled awnless. Lower glume of sessile spikelet broad, the joints and pedicels solid.

Racemes shortly peduncled, usually sub-digitate .. .. .. *Dichanthium*

CCC Spikelets in threes, one sessile along with two pedicelled at the ends of branches of a terminal panicle .. .. *Chrysopogon*

CCCC Pedicelled and sessile spikelets on a short rachis, subtended by an involucre of four pedicelled spikelets. Inflorescence a panicle bearing spathe-like bracts .. .. *Iseilema*

BB Internodes or pedicels not filiform.

C Raceme reduced to one sessile spikelet and two strap-like pedicels, one bearing a spikelet and the other a solitary minute glume (reduced spikelet). Each raceme in a spathiform bract, the inflorescence paniculate, leafy .. *Apluda*

CC Raceme spiciform, its rachis bearing the pedicelled and sessile spikelets in pairs but the pedicels fused with the internodes and the sessile spikelets sunk in concavities of the internodes. Rachis disarticulating tardily. Inflorescence of spathe-supported racemes .. *Hemarthria*

AAAAA Spikelet one-flowered, strongly laterally compressed, the glumes very minute or confluent into an annular rim at the tip of the pedicel. Sterile lemmas above the articulation of the pedicel 2, very small or subulate, or sometimes 0. Fertile lemma large, chartaceous or coriaceous, sometimes awned; the palea as long as the lemma, coriaceous. Inflorescence a panicle .. .. *Oryza*

ENUMERATION OF THE SPECIES COLLECTED

Apluda mutica L. var. *aristata* Pilger
Apluda varia Hack. subsp. *aristata* Hack.
Waste ground east of Carlton Hotel, 25-10-1957.
PATIL, 481, 482.

Bothriochloa pertusa A. Camus
Amphilophis pertusa Stapf
Roadside, Amausi Airfield, 30-8-1957.
PATIL, 451.

Brachiaria ramosa Stapf
At the border of a cultivated field, Bangla Bazaar, 24-9-1958.
E. K. J., 7204, PATIL, 1711.

Cenchrus ciliaris Linn.
Waste area, Carlton Hotel, 12-7-1956.
E. K. J., 7211.

Chrysopogon aciculatus Trin.
On moist ground near the lake, Chinhat, 25-9-1958.
PATIL, 1713.

Cynodon dactylon Pers.
Roadside, Amausi Airfield, 30-8-1957.
E. K. J., 7209, PATIL, 452.

Dactyloctenium ægyptium P. Beauv.
Roadside, Amausi Airfield, 30-8-1957.
E. K. J., 7205, PATIL, 450.

Desmostachya bipinnata Stapf
Near Amausi Airfield, 24-5-1957.
E. K. J., 7517.

Dichanthium annulatum Stapf
Near Amausi Airfield, 25-4-1957.
E. K. J., 7522.

Digitaria sanguinalis Scop.
Digitaria marginata Link.
Digitaria adscendens Henr.
Border of a cultivated field near Amausi Airfield, 23-9-1958.
E. K. J., 7193, PATIL, 1704.

Digitaria granularis Henr.
Digitaria pedicellaris Prain
Border of a cultivated field near Amausi Airfield, 23-9-1958.
PATIL, 1705.

Echinochloa colona Link.
As weed in National Botanic Gardens, 21-9-1957.
PATIL, 469.

Echinochloa crusgalli P. Beauv.
On wet ground, lakeside, Chinhat, 25–9–1958.
PATIL, 1712.

Erianthus munja Jeswiet
Saccharum munja Roxb.
In hedges near Chinhat Lake, 29–11–1958.
PATIL, 1289.

Eleusine indica Gaertn.
Waste area, Carlton Hotel, 11–7–1956.
E. K. J., 7196.

Eleusine verticillata Roxb.
Right bank of the Gomti, 16–7–1956.
E. K. J., 7217.

Eragrostis tenella Beauv. var. *plumosa* Stapf
As weed in National Botanic Gardens, 9–10–1957.
PATIL, 476.

Eragrostis poæoides Beauv.
Eragrostis minor Host.
Near Amausi Airfield, 25–5–1957.
E. K. J., 7518.

Eriochloa procera C. E. Hubb.
Eriochloa ramosa Kuntze
Wet ground, lakeside, Chinhat, 25–9–1958.
PATIL, 1715.

Hemarthria compressa R. Br.
Border of a pool, Bangla Bazaar, 24–9–1958.
E. K. J., 7208, PATIL, 1709.

Imperata cylindrica P. Beauv.
Waste area, Carlton Hotel, 11–7–1956.
E. K. J., 7200.

Iseilema laxum Hack.
Roadside, Amausi Airfield, 30–8–1957.
PATIL, 454.

Leptochloa chinensis Nees
On wet ground, lakeside, Chinhat, 25–9–1958.
PATIL, 1716.

Leptochloa filiformis Roem. and Schult.
Waste area, Carlton Hotel, 11–7–1956.
E. K. J., 7202.

Oplismenus burmanii P. Beauv.
Shady lawn, National Botanic Gardens, 7–10–1957.
PATIL, 475.

Oryza sativa Linn. var. *fatua* Prain (Wild Rice).
In roadside ditches along Sitapur road, during the rains.
CBL, 73.

Panicum antidotale Retz.
Right bank of the Gomti, 27–6–1956.
E. K. J., 7186.

Paspalidium flavidum A. Camus
On high ground near Chinhat Lake, 25–9–1958.
PATIL, 1714.

Paspalum distichum Linn.
Border of a cultivated field, Bangla Bazaar, 24–9–1958.
E. K. J., 7192, PATIL, 1708.

Pseudoraphis squarrosa Chase
Pseudoraphis aspera Pilger
In a pool, Bangla Bazaar, 24–9–1958.
PATIL, 1710.

Saccharum spontaneum Linn.
On low-lying, watery ground between Lucknow and Amausi, 5–9–1957.
PATIL, 456.

Setaria glauca Beauv.
Roadside, Amausi Airfield, 30–8–1957.
PATIL, 453.

Setaria tomentosa Kunth.
Setaria intermedia Roem. and Schult.
Roadside, Amausi Airfield, 23–9–1958.
PATIL, 1706.

Setaria verticillata Beauv.
Waste area, Carlton Hotel, 11–7–1956.
E. K. J., 7206.

Sorghum halepense Pers.

In sugarcane field near the Sugarcane Research Institute, 13–8–1957. E. K. J., 7210, PATIL, 430.

Sporobolus diander Beauv.

Waste area, Carlton Hotel, 11–7–1956. E. K. J., 7191.

Urochloa helopus Stapf

Near Amausi Airfield, 25–5–1957. E. K. J., 7523.

SUMMARY

An enumeration is given of the common grasses of Lucknow and its environs with notes on locality of occurrence, habitat and the date of collection in the flowering state. Attention is directed to recent nomenclatural changes. A generic key based on the current classification is presented for the ready identification of the common Lucknow genera.

ACKNOWLEDGEMENTS

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FACTORS INFLUENCING ENDOTROPHIC SPORULATION IN AEROBIC SPOREFORMING BACILLI

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The ability of certain bacterial species to produce resistant endospores has since the early days of bacteriology engaged the attention and energies of bacteriologists, some of whom have been great minds of the day (notably Pasteur, Ehrenberg and Cohn). The fact that in spite of a century of research, this unique biological structure still invites investigation, is eloquent testimony of the problems that it still holds for us. Studies on the physiology of bacterial sporulation have two rewarding aspects. The first and the more stimulating is the positive attempt that it makes to answer the fundamental question as to why a bacterium forms a spore. The second and perhaps the more useful aspect is the application which such knowledge would have on the control of sporulation and therefore on the control of spoilage in several industries where heat-resistant sporeforming bacilli constitute a major threat (Iyer and Bhat, 1952).

While the conditions necessary for the formation of bacterial endospores have been much debated, it has been suspected in recent times that sporulation in bacteria is an event that can occur in the absence of multiplication and in media devoid of nutrients (Hardwick and Foster, 1953; Perry and Foster, 1954). In experiments conducted in this laboratory (Iyer, unpublished data), evidence was obtained for the conclusion that sporogenesis could be an endogenous event which can occur when a vegetative cell is placed in distilled water. In this paper are presented the results of further experiments on the extent to which this phenomenon occurs among aerobic sporeforming bacteria, and the factors influencing it.

Materials and Methods

Species and strains.—Five strains each of *Bacillus subtilis, B. cereus, B. megaterium, B. circulans, B. firmus* and one strain of *B. sphæricus* were used in the initial experiments to establish the extent to which endotrophic

sporulation occurs among species of aerobic sporeforming bacteria. Taken together, these species constitute the dominant and most frequently occurring members of the genus (Bhat and Iyer, 1955). For further experiments, one strain of *B. cereus* showing maximum endotrophic sporulation and one strain of *B. subtilis*, showing scant sporulation, were selected.

Preparation of inoculum.—Six to seven-hour old cultures from nutrient agar (pH 7·0) were used as the source of vegetative cells. Within this period, none of the strains under investigation had begun to sporulate. The growth from the surface of the medium was washed three times with sterile glass-distilled water before being used as the inoculum. The complete absence of spores from such suspensions was checked by microscopically examining monochrome (crystal violet) stained smears.

Estimation of sporulation.—The criteria that have been used as a measure of the degree of sporulation are heat resistance (Curran and Evans, 1954), optical density (Hachisuka *et al.*, 1954) and a decrease in the ability to take up stains (Roberts and Baldwin, 1942; Foster and Helligman, 1949; Williams and Lamanna, 1952). Among these, the simpler and more convenient morphological methods have found favour with many contemporary workers (Weinberg, 1955; Amaha, 1956; Krishna Murthy and Halvorson, 1957). In this laboratory, the extent of sporulation was followed using four methods and complete correlation in the results were obtained (Kanga, 1959). Monochrome staining with crystal violet was the method of choice. A total of 300 cells was counted in different areas of a smear before arriving at the percentage of cells having undergone sporulation.

Compounds tested for their effect on endotrophic sporulation.—The compounds that were tested for their ability to inhibit or enhance endotrophic sporulation included metallic salts, carbohydrates, organic acids, amino-acids and growth factors. The metallic salts (except manganese chloride), carbohydrates, and organic acids (sodium salts) were sterilized by autoclaving. Manganese chloride, the amino-acids and growth factors were sterilized by Seitz filtration.

EXPERIMENTAL RESULTS

Species and strain differences.—The degree of endotrophic sporulation observed with the various strains and species tested is presented in Table I. Under the conditions of the experiment, it was observed that strains of *B. megaterium* invariably underwent a concurrent lysis in distilled water. Since the lysed products may possibly serve as exogenous nutrients, experiments with this species were discontinued. No lysis was observed with any

TABLE I

Endotrophic sporulation among sporeforming bacilli—species and strain differences

Strains and species			Per cent. sporulation in distilled water after 5 days
		B. subtilis	
Strains	(1)	..	10
	(2)	..	25
	(3)	..	35
	(4)	..	25
	(5)	..	7
		B. cereus	
Strains	(1)	..	50
	(2)	..	85
	(3)	..	60
	(4)	..	46
	(5)	..	50
		B. circulans	
Strains	(1)	..	0
	(2)	..	0
	(3)	..	0
	(4)	..	0
	(5)	..	0
		B. firmus	
Strains	(1)	..	20
	(2)	..	25
	(3)	..	20
	(4)	..	7
	(5)	..	15
		B. sphæricus	
Strain	(1)	..	25

of the strains of all the other species tested. The degree of endotrophic sporulation observed varied with both strains and species. Maximum sporulation was observed with strains of *B. cereus* while strains of *B. circulans* were unable to sporulate in five days. A strain of *B. cereus* showing maximum sporulation was selected to study the ability of various exogenous substances to inhibit sporulation. Likewise, a strain of *B. subtilis* showing slight sporulation was selected for studies on factors enhancing sporulation.

Effect of metallic salts.—In preliminary experiments using 0·1 and 1·0 molar concentrations, several of the metallic salts tested were found to inhibit endotrophic sporulation, the pattern of inhibition being identical with the two strains used. *B. cereus*, being the more suitable for the study of the inhibition phenomenon, was selected for further studies using the inhibitory salts in the concentration range of 1–1,000 mg. per cent. The effect of these salts on the growth of the strain in nutrient broth was also studied. The results presented in Table II indicate that the inhibition of growth in nutrient broth generally occurs at slightly higher concentrations than those inhibiting endotrophic sporulation.

Effect of amino-acids.—All the essential amino-acids as well as asparagine were tested out at a concentration corresponding to 25 mg. per cent. nitrogen. Only valine, isoleucine, methionine and asparagine effectively inhibited sporulation in the two test strains. Studies with these four amino-acids were therefore extended to other strains of sporeforming bacilli and the results are summarized in Table III. It is observed that the inhibitory effect is neither general nor characteristic of a species but is strain specific. None of these amino-acids inhibited growth when tested in nutrient broth at concentrations upto five times the concentration used for sporulation studies.

Effect of organic acids.—The sodium salts of the following organic acids were tested: formic, oxalic, benzoic, salicylic, tartaric, oleic and propionic (all at 0·1 per cent. concentration) and citric acid (0·5 per cent.). None of the compounds significantly favoured or inhibited sporulation. Since at higher concentrations, formate, oxalate, benzoate, salicylate and tartrate are known to be inhibitory to growth, the effect of these salts on sporulation and growth in *B. cereus* was studied. The results appear in Table IV. As in the case of metallic salts, it was found that growth inhibitory concentrations of the organic acids or concentrations slightly lower also inhibited sporulation.

Effect of carbohydrates.—The following carbohydrates were tested at a concentration of 0·5 per cent.: xylose, glucose, fructose, galactose, sucrose, maltose, inulin, glycerol and mannitol. Of these, xylose and the hexoses,

TABLE II

Effect of metallic salts on endotrophic sporulation and growth of Bacillus cereus (*Strain* 2)

Metallic salts	Concentration (mg. per cent.)	Per cent. sporulation (6 days)	Degree of growth*
Distilled water (control)	..	85	..
Nutrient broth (control)	..	..	3
Cobalt sulphate ..	1	85	3
	10	30	3
	100	0	0
	1000	0	0
Copper sulphate ..	1	0	3
	10	0	3
	100	0	1
	1000	0	1
Magnesium sulphate..	1	90	2
	10	80	3
	100	77	3
	1000	60	3
	10000	55	3
Manganese chloride ..	1	80	3
	10	95	3
	100	0	3
	1000	0	1
Nickel chloride ..	1	10	3
	10	0	3
	100	0	0
	1000	0	0
Potassium chloride ..	745·5 (0·1 M)	90	3
	7455·0 (1·0 M)	92	3
Sodium chloride ..	584·5 (0·1 M)	65	3
	5845·0 (1·0 M)	90	3
Zinc sulphate ..	1	65	3
	10	7	2
	100	0	1
	1000	15	1

* 0 = less than 10^1 cells/ml.
1 = 10^1 to 10^6 cells/ml.
2 = 10^6 to 10^7 cells/ml.
3 = greater than 10^7 cells/ml.

TABLE III

Effect of isoleucine, valine, methionine and asparagine on the endotrophic sporulation of sporeforming bacilli

Strains and species		Per cent. sporulation after 6 days in				
		Distilled water	Dl-Isoleucine	Dl-Valine	Dl-Methionine	L-Asparagine
		B. subtilis				
Strains	(1) ..	10	0	11	2	0
	(2) ..	30	27	40	0	25
	(3) ..	30	41	25	15	28
	(4) ..	35	7	15	10	30
		B. cereus				
Strains	(1) ..	75	50	40	65	80
	(2) ..	80	45	0	3	38
	(3) ..	60	55	40	10	55
	(4) ..	55	35	30	42	50
	(5) ..	50	30	40	66	40
		B. firmus				
Strains	(1) ..	25	30	28	0	20
	(2) ..	25	30	16	0	15
	(3) ..	5	20	6	0	0
		B. sphæricus				
Strain	(1) ..	15	7	7	0	10

particularly the latter, significantly inhibited sporulation (more than 50 per cent.). Concentrations between 0·05 and 0·5 per cent. were effective, the inhibition increasing progressively above that concentration. In further experiments with *B. cereus*, the effect of the addition and removal of the three hexoses after sporulation in distilled water had begun, was studied, the removal being accomplished by centrifugation and washing (*see* Figs. 1 and 2). It was observed that the inhibition of sporulation occurred even when the compounds were added after the process had started. Likewise, when the sugars were removed from the distilled water half-way through sporulation, the rate was immediately enhanced and caught up with the control.

In the experiments described, the hexoses were sterilized by autoclaving concentrated aqueous solutions and later diluting to the desired concentra-

TABLE IV

Effect of the sodium salts of some organic acids on endotrophic sporulation and growth in Bacillus cereus (*Strain* 2)

Sodium salts	Concentration (gm. per cent.)	Per cent. sporulation in 5 days	Degree of growth in nutrient broth*
Distilled water (control)	..	80	..
Nutrient broth (control)	..	..	3
Formate ..	0·1	30	1
	1·0	55	1
Oxalate ..	0·1	65	2
	1·0	18	2
Benzoate ..	0·1	10	2
	1·0	0	1
Salicylate ..	0·1	10	2
	1·0	25	0
Tartrate ..	0·1	75	2
	1·0	30	2

* 0 = less than 10^1 cells/ml.
1 = 10^1 to 10^6 cells/ml.
2 = 10^6 to 10^7 cells/ml.
3 = greater than 10^7 cells/ml.

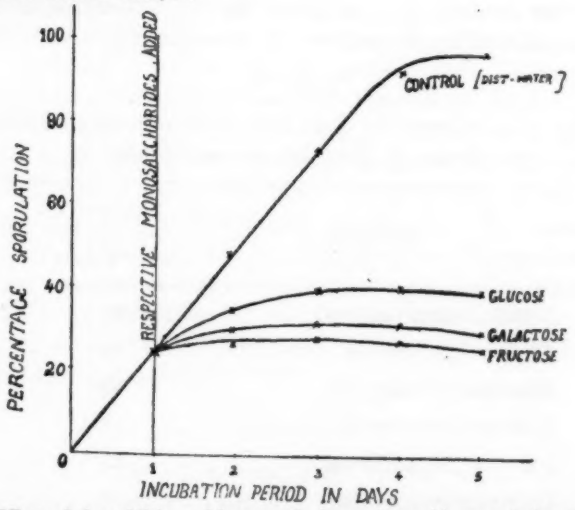

FIG. 1. Effect of the addition of hexoses during endotrophic sporulation in *Bacillus cereus* (Strain 2).

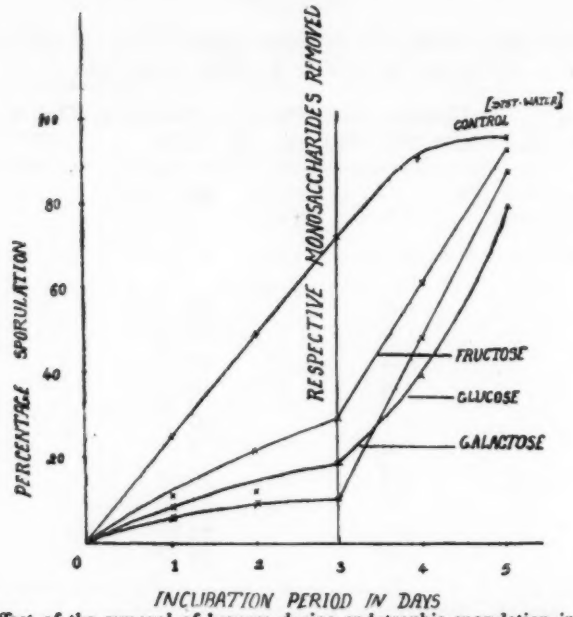

FIG. 2. Effect of the removal of hexoses during endotrophic sporulation in *Bacillus cereus* (Strain 2).

tion. When the hexoses were sterilized by Seitz filtration and used, the inhibition was reduced by 50 per cent. or more (*see* Table V). This suggests

TABLE V

Effect of autoclaved and Seitz filtered hexoses on endotrophic sporulation in Bacillus cereus (*Strain* 2)

Hexoses	Per cent. sporulation after 5 days
Distilled water (control) ..	80
Glucose (autoclaved) ..	10
Glucose (Seitz filtered) ..	40
Fructose (autoclaved) ..	25
Fructose (Seitz filtered) ..	60
Galactose (autoclaved) ..	5
Galactose (Seitz filtered) ..	70

that during the autoclaving of even concentrated sugar solutions (a hitherto accepted procedure), significant changes do take place and the products act differently from Seitz-filtered sugars.

Effect of growth factors.—The effect of the following growth factors was tested: pyridoxine, pantothenic acid, folic acid, uracil, adenine (0·1 μg./ml.), cytosine (0·01 μg./ml.), thiamine (0·01 μg./ml.), biotin (0·0001 μg./ml.) and vitamin B_{12} (10^{-6} μg./ml.). None however had any effect on endotrophic sporulation.

Discussion

Despite early scepticism of observations on the ability of a vegetative cell to sporulate in a non-nutrient medium, this is now a confirmed and well-authenticated phenomenon for most species of aerobic sporeforming bacilli. While our results suggest that strain differences do exist with respect to the extent of endotrophic sporulation, it would appear that in some species like *B. cereus*, this occurs more readily than in others like *B. circulans*. It has been suggested that certain constituents of the vegetative cells, probably enzyme proteins, are degraded and resynthesized into the materials characteristic of the spore (Hardwick and Foster, 1953; Foster and Perry, 1954). Environmental agents that cannot support growth can however influence endotrophic sporulation, presumably by affecting the metabolic steps leading to the biosynthesis of the spore. While none of the compounds tested were able to significantly increase the degree of endotrophic sporulation, several of them were found to inhibit the process. The agents that inhibit endotrophic sporulation include several metallic salts, amino-acids, fatty acids and autoclaved carbohydrates. With several of these compounds it was observed that concentrations that were growth inhibitory or concentrations slightly below this, also inhibited endotrophic sporulation. This suggests that all or most growth inhibitory substances may at similar or lower concentrations, also inhibit endotrophic sporulation. The converse of this concept is however not true, that is, the inhibition of sporulation by concentrations of a compound may occur without a corresponding inhibition of growth. This is illustrated by the four amino-acids which do not inhibit growth even at concentrations five times greater than concentrations that suppress endotrophic sporulation.

Summary

The ability of strains of representative species of sporeforming bacilli to sporulate endotrophically in distilled water has been demonstrated. The degree of sporulation which a bacterium may undergo is a strain specific

character but some species are more capable than others. The effect of various exogenous compounds on the phenomenon has been studied. It has been observed that various metallic salts, organic acids and autoclaved carbohydrates inhibit sporulation at concentrations that are growth inhibitory in nutrient media. It has been postulated that all or most growth inhibitory substances may at similar or lower concentrations inhibit endotrophic sporulation. A hitherto accepted procedure of sterilisation, the autoclaving of concentrated aqueous solutions of sugars, has been found to enhance the ability of the sugar to inhibit sporulation. None of the growth factors tested had any effect on sporulation.

ACKNOWLEDGEMENTS

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DRUG RESISTANCE IN YEAST (*SACCHAROMYCES CEREVISIAE*) ACTION OF GENTIAN VIOLET

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Introduction

Hinshelwood *et al.*[1] have demonstrated that the gradual development of training of yeast cells is more likely to be an adaptative response to the inhibitor than the selection of preformed mutants. It is, therefore, indicated that in a resistant strain developed due to drug action the adapted cells are in preponderance over the mutant cells.

The authors have studied the effect of Gentian violet on yeast cells and have already reported[2] that when a resistant strain of *Saccharomyces cerevisiæ*, presumably a mixture of adapted and mutated cells as developed by the action of Gentian violet is plated on drugged agar medium two types of colonies appear. The characteristic electrode potentials of these colonies have already been discussed in a previous communication.[3] In this paper the growth characteristics of primary and secondary colonies are reported. An attempt has been made to further differentiate between the cells arising out of the two types of colonies.

Experimental

The strain employed was *Saccharomyces cerevisiæ* (Hansen, I.I.S.T. No. 13). It was trained[2] by growing in synthetic full rich medium according to White and Munns[4] which had the following composition and to which increasing concentrations of Gentian violet were added as the resistance increased:—

Ammonium sulphate	4 g.
Potassium sulphate	4 g.
Disodium mono-hydrogen phosphate	2 g.
Magnesium sulphate	0·5 g.
Calcium chloride	0·1 g.

Ferric ammonium sulphate	0·02 g.
Zinc sulphate	0·2 g.
Copper sulphate	0·01 mg.
Thiamine hydrochloride	200 micro gram
Riboflavin	100 „
Nicotinic acid	5000 „
p-Amino benzoic acid	300 „
Pyridoxine hydrochloride	1000 „
Calcium de-pantothenate	500 „
m-Inositol	50000 „
d-Biotin	6 „
Sucrose	100 g.
Distilled water	1000 ml.
pH	4·6

In subsequent experiments also the same synthetic medium with appropriate concentrations of the drug was used. The temperature of incubation was $32 \pm 0{\cdot}1°$ C. A twenty hours old culture in the logarithmic phase was always employed in these experiments. All experiments were performed at least in duplicates.

Isolation of two types of colonies from the resistant strain

The nutrient medium with a Gentian violet concentration of 8 mg./litre was solidified with 1·5% agar. The medium after sterilization was inoculated with different concentrations of cells from the resistant strain and incubated. Large, star-shaped primary colonies and small pin-point secondary colonies were isolated from the same plate after six days of incubation and preserved on agar slants for further studies. These were renewed fortnightly.

In the manometric determinations growing cells from the two types of colonies were employed. Since it was observed that secondary cells have a higher cell mass synthesizing capacity and we were interested in comparing the oxygen uptake and carbon dioxide output as the cells proliferated resting cells were not used.

(*i*) *Rate of carbon dioxide evolution.*—This was measured according to the "Direct method" for carbon dioxide[5] in a Warburg's respirometer. The main compartments of the Warburg's flasks contained 1·8 ml. of the

drugged or drug-free nutrient medium. To the side arm of the flasks was added a uniform cell suspension (0·2 ml. in phosphate buffer; pH 4·6) representing $5{\cdot}5\times10^6$ cells/ml. The flasks were attached to the manometers and placed in the Warburg bath maintained at $35 \pm 0{\cdot}03°$ C. The atmosphere in the flasks and manometers was saturated with oxygen-free carbon dioxide to obtain anaerobic conditions by passing carbon dioxide through a manifold to the Warburg flasks which were shaking in the bath. The contents of the side arm were then fitted into the main compartment and the rate of anaerobic fermentation measured in terms of carbon dioxide evolution.

Figure 1 shows the $ulCO_2$ evolved per minute.

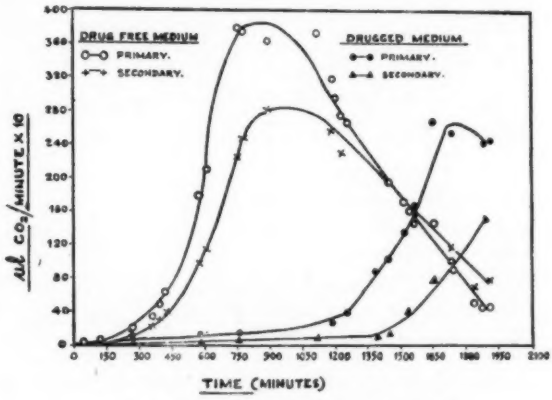

FIG. 1. $ulCO_2$/minute-time relationship.

In case of the medium without drug the rate of evolution of carbon dioxide is greater in the primary colonies. The secondary colony cells show a delayed evolution of carbon dioxide. In the drugged medium this delay becomes more pronounced.

(*ii*) *Oxygen uptake.*—Oxygen absorption was measured[6] by setting up of the Warburg flasks as described in the case of the measurement of carbon dioxide evolution. The contents of the flasks were exactly the same except that the central cup in this case contained 0·2 ml. of 20% KOH with a strip of filter-paper dipped in it to provide a large surface. Air was allowed to remain in the flasks to provide oxygen for respiration and no carbon dioxide was passed in this case.

Figures 2 *and* 3.—Figures 2 and 3 give the oxygen uptake by the cells from the two types of colonies as well as by the untrained cells. The requirements of secondary colony cells as regards their oxygen uptake are relatively

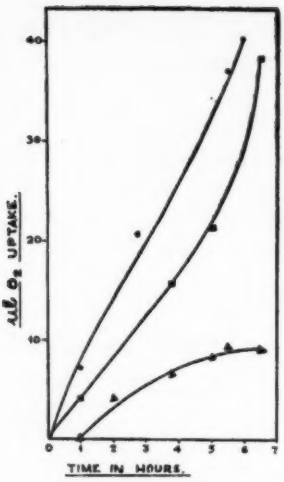

FIG. 2

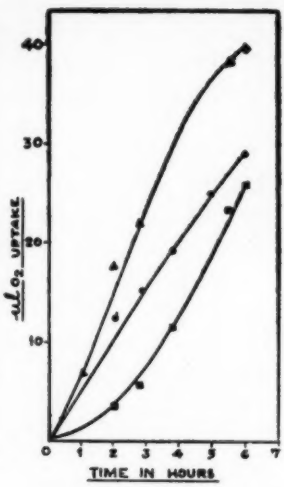

FIG. 3

FIG. 2. Oxygen uptake in drug-free medium:
▲——▲, untrained cells; ♦——♦, primary colony cells;
●——●, secondary colony cells.

FIG. 3. Oxygen uptake in the drugged medium:
▲——▲, untrained cells; ♦——♦, primary colony cells;
●——●, secondary colony cells.

large. In the drugged medium (Fig. 3), however, it is the untrained cells that consume more oxygen. The primary colony cells show lesser uptake both in the drugged and drug-free medium as compared with the secondary colony cells.

(*iii*) *Growth in terms of turbidity*.—Equal volumes of the drugged medium were inoculated with a uniform cell suspension of $1{\cdot}6\times10^7$ cells/ml. from primary, secondary and untrained colony cells. As the growth proceeded aliquot samples were drawn out from time to time for measurement of turbidity. The cells were spun down at 3,000–3,500 r.p.m., quantitatively removed and suspended in equal volumes of phosphate buffer. The percentage absorption of these cell suspensions was measured in a 2 cm. cell using a Spekker's absorptimeter.

Figure 4 *shows the growth*.—The secondary colony cells recorded a higher rate of growth as compared with primary and untrained cells.

(*iv*) *Alcohol forming capacity*.—The initial sugar concentration in the flasks set up was kept constant. Inocula in all cases were $2{\cdot}85\times10^7$ cells/ml.

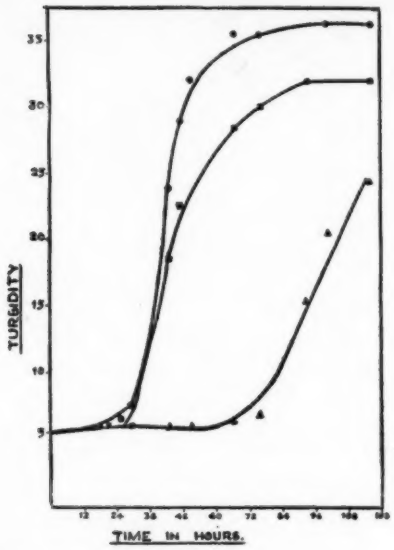

FIG. 4. Growth in terms of turbidity:

▲——▲, untrained cells; ♦——♦, primary colony cells; ●——●, secondary colony cells.

30 ml. of the fermented liquid was diluted, distilled and made up to a definite volume in each case. The alcohol in the total distillate was estimated by oxidizing with potassium dichromate and titrating the residual chromic acid iodometrically by the method of Martini.[7]

Table I gives the alcohol formed.

TABLE I

Alcohol formed gm./100 ml. (120 *hrs.*)

Description	Drug-free medium	Drugged medium
Primary colony cells ..	4·4 1·035	3·9 11·015
Secondary colony cells ..	2·2 1·040	3·8 1·040

(*v*) *Cell mass synthesized.*—Aliquots of 100 ml. of drugged and drug-free medium were inoculated with an equal and uniform suspension of primary

and secondary colony cells. The yeast formed after completion of the fermentation was centrifuged, washed, dried in vacuum at 60·0° C. and weighed. It is found that the secondary colony cells synthesize more cell mass than the primary colony cells (Table II).

TABLE II

Dry yeast formed gm./100 ml.

Description	Drug-free medium 120 hours	Drugged medium 170 hours
Primary colony cells	0·1456	0·1800
Secondary colony cells	0·1842	0·1906

DISCUSSION

Hinshelwood *et al.*[8] have reported that secondary colonies isolated from yeast as a result of the action of crystal violet, utilise glucose in a relatively wasteful manner and that their cell synthesizing capacity is less than that of the primary colonies. They have further shown that the secondary colony cells take a longer time in reducing tetrazolium chloride. On the basis of above evidence it is advocated that some oxidation-reduction systems are impaired.

Less alcohol yields (Table I) and poorer evolution of carbon dioxide (Fig. 1) from the secondary colony cells indicate that some reductase system in the decarboxylation of pyruvic acid to acetaldehyde and its subsequent reduction to alcohol is interfered with.

The present authors have already reported[3] that the electrode potentials in growing secondary colony cultures are higher than those of the primary colony cells. Since electrode potential is a measure of the ratio of the oxidized to the reduced form, it is evident, therefore, that the oxidized forms of the substrates are more abundant in the secondary colony cultures. The relatively higher rate of oxygen uptake (Figs. 2 and 3) by the secondary colony cells supports the above observation that the conditions therein tend to be oxidative. In view of the fact that reduction system is impaired a comparatively more oxidizing system results. This accounts for the higher oxygen uptake by the secondary colony cells which results in the synthesis of more cell mass (Table II).

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SUMMARY

A resistant strain of yeast when plated on drugged agar medium gives rise to primary and secondary colonies. A comparative study of the two types of cells from these colonies has been reported in regard to carbon dioxide evolution, oxygen uptake, alcohol forming capacity and cells mass synthesized. The secondary colony seems to arise from a mutant strain.

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ON THE ROOT ROT OF *KOCHIA INDICA* WIGHT

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Introduction

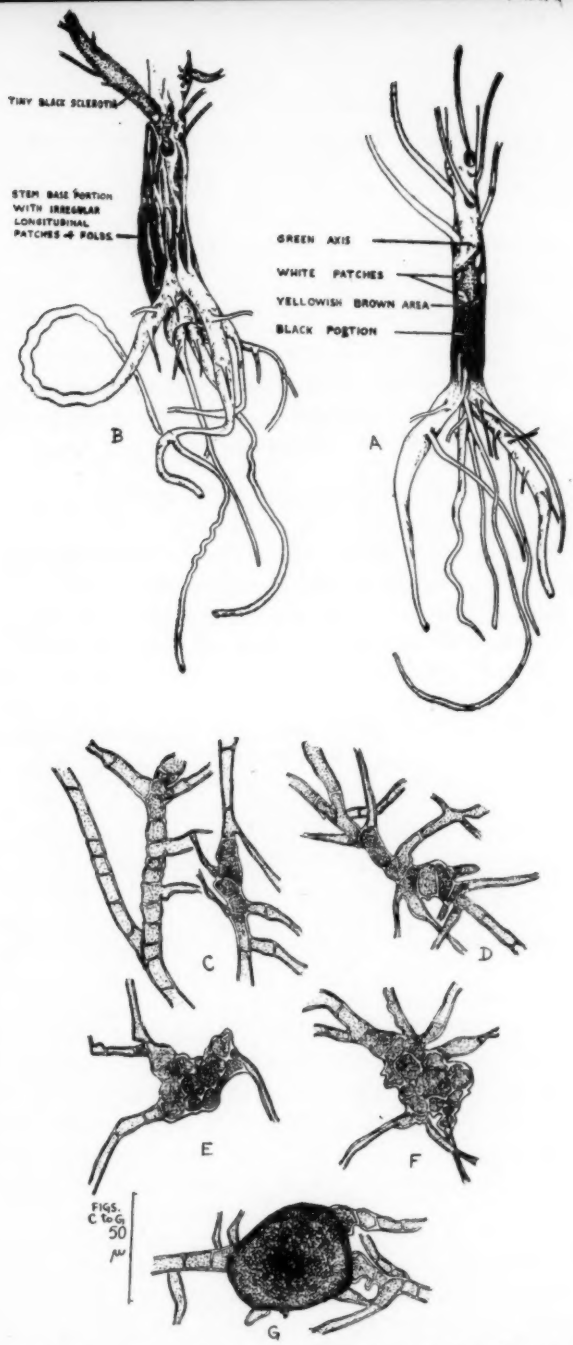
Kochia indica Wight (Chenopodiaceæ), ornamental plants of the summers suffer regularly from a 'root rot' disease which is found to occur in almost all the Lucknow gardens. The diseased parts of the plants yielded only two fungi: a species of *Fusarium* sp. and *Rhizoctonia solani* Kuhn. Of these the latter has been found to be dominantly associated with the affected parts showing different stages of the disease while the former only with the root and the stem base of plants exhibiting advanced symptoms.

The preliminary experiments showed that only *R. solani* was responsible for the causing of the root rot in *Kochia indica* while *Fusarium* sp. is associated only as a secondary fungus being a soil dweller. The present paper gives a brief account of the various pathological aspects of this disease.

Symptoms

The earliest symptom of the root rot in young plants is the etiolation of the stem base which gradually turns brown and at the ground level becomes black while at the top it merges into the healthy green colour of the stem. Some scattered white spots appear in the etiolated portions and later the foliage dries up (Text-Fig. 1, *a*).

When the infection occurs in older plants, the withering of the foliage starts from the lowermost branches where the leaves at the base are completely dried up while those at the top exhibit only initial signs of drying. The withered leaves show a downward curl from their apices and after the shoot has dried up, they fall off within a fortnight. The main axis gradually turns reddish-brown, becomes hard and remains erect even in the fully withered plants. The basal portion of the stem develops several irregular longitudinal folds (Text-Fig. 1, *b*); which at the ground level assumes dark colour, increases in diameter and starts rotting emanating a characteristic odour. The rotting also involves the upper portion of the roots. The cortical layers



TEXT-FIG. 1. A, B — Diagrammatic presentation of the diseased *Kochia* plants. (A) Showing early stage in the development of disease. (B) Showing advanced stage of the disease. (C to G) Camera lucida sketch to illustrate the different stages in the formation of the selerotium.

including the bast of the rotted portions can easily be peeled off. Some of the roots, in advanced stages of the disease, are found to have irregular wavy outlines.

In the completely dried up plants, towards the final stage of the disease, a large number of tiny black sclerotia appear all over the outer surface of the branches, which gradually involves the main axis and also some of the roots (Text-Fig. 1, *b*).

No galls or tumours are developed on the root or the shoot of the affected plants at any stage of the development of the disease. The internal symptoms have been described elsewhere (Histopathology).

PATHOGENICITY

A large number of seedlings, raised from healthy *Kochia* seeds, were transplanted in pots containing sterilised soil. Plants between 1 to 4 months of age were normally employed for the experiments, and the inoculum was taken from 6 to 8-day old bacteria free monohyphal culture of *R. solani*. The method of experiment consisted in the inoculation of the root and the hypocotyl region of the seedlings. In the former the seedling root was either directly inoculated with and without wound, or, the inoculum was mixed with the soil of the rhizosphere; while in the latter the inoculum was applied on the hypocotyl region.

The infection attempted through the wounded and unwounded hypocotyl proved unsuccessful for *Kochia* plants of any age, whereas the root inoculation gave successful results.

In direct inoculations on the root, the one-month old seedlings succumb readily in three days after the experiment (90%). The top portion of the shoot becomes flaccid, collapses down forming an arch and dries up within a week (Plate VII, Fig. 1). The older seedlings, however, show a gradual withering of the foliage. In the experimented plants of 2–3 months age, the first visual sign of infection is noted after three weeks of the experiment and complete withering within a fortnight from this in 40–60% cases (Plate VII, Fig. 2). The injury did not in any way seem to favour the infection as the inoculations on the experimentally injured roots yielded infection in not more than 50% plants.

In the rhizosphere inoculation the disease symptoms were visible generally in the third week after the inoculation and 30% plants dried up completely after six weeks, except in the case of one-month old seedlings where the percentage of the rot produced was 60%.

The fungus employed for the artificial inoculation of the plants was reisolated from the experimentally diseased plants in the usual manner.

Morphology of the Pathogen in Culture

Hyphæ form a hallow round the inoculum when young, are 2·5 to 3·0 μ wide. With age turn reddish-brown, branches arising at right angles to parent hypha and bending after a short length becoming nearly parallel to it, 9·3 to 12·0 μ wide and the septa being 13·8 to 24·8 μ apart. In old cultures a white hyphal tangle is developed aerially but the sclerotia are not produced in this aerial mass.

The sclerotia are formed after the fourth day of inoculation on thick brown hyphæ which divide into rectangular cells measuring 9·0 to 9·3 $\mu \times$ 12·4 to 18·6 μ. Some cells branch outwards, are barrel-shaped and measure 15·5 $\mu \times$ 30·0 μ. The hyphæ are attached as fibrils all round the sclerotium, the average dimension being 56·0 to 63·0 $\mu \times$ 84·0 to 85·0 μ and 112·0 $\mu \times$ 182·0 μ. Some are roundish in structure and measure 154·0 $\mu \times$ 154·0 μ (Text-Fig. 1, C to G).

The cardinal temperatures for this strain of *R. solani* have been determined as: minimum 10° C., maximum 40° C. and optimum 25 to 30° C.

Histopathology

A study of the histopathology of the various parts of the diseased plants, collected from nature as well as those produced experimentally, using the usual technique, gives a complete picture of the host invasion by the fungus from the root through the hypocotyl region into the stem and branches of the host tissue.

As the pathogen penetrates through the epidermis in the seedling root or in younger roots of the more mature plants, the hyphæ remain restricted to the epidermal and subepidermal regions. The sclerotia if formed are lodged in the epidermis. The fungus gradually encroaches upon the xylem vessels and as the disease progresses and more sclerotia are developed in the vascular zone, the central cylinder which was intact in the early stages, breaks up into 3 to 4 concentric incomplete rings. The sclerotia fill up these gaps of the separated vascular rings and the accompanying dark hyphal tangle spreads over the vessels of either side (Plate VII, Fig. 3). Relatively only a few vessels are found to enclose the sclerotia which measure 42 $\mu \times$ 28 μ to 84 $\mu \times$ 42 μ. The hyphæ present in the xylem vessels of the innermost ring are lighter in colour than those distributed towards the periphery where they are

arranged in a radial manner between the vessels. This radial arrangement of the hyphæ is more pronounced in the upper regions of the infected plants.

From the root, with the advance of the disease, the pathogen enters the hypocotyl and the stem base region, spreading copiously in the cortical as well as in the central cylinder. The peripheral ground tissue becomes rotted and forms a weak covering over the stele in which the hyphæ are arranged radially between the vessels. Generally a few sclerotia and hyphæ fill these vessels and also the small cavities developed in between the xylem rings of the stele which are not as completely separated as in the mature root.

Further up in the region of the central axis below the level of lateral branches, the fungus spreads all over the general tissue but more towards the centre even involving a few cells of the pith. There is no marked breaking of the stele in this region, but few sclerotia are found lodged in between the xylem rings and the hyphæ are conspicuously arranged in the characteristic radial manner over the stele (Plate VII, Fig. 4).

The pathogen, while entering into the branches, ramifies profusely all over the cortical and subepidermal regions mainly as inter- but also as intracellular hyphæ which are 3·5 to 6·2 μ wide and form a thick mat between the cortex and the stele. In contradistinction with other parts the hyphal distribution over the vascular cylinder here is scattered. The rounded or elongate sclerotia are developed only in the extrastelar tissue, their average dimensions being, 98 $\mu \times 112\,\mu$ to 140 $\mu \times 175\,\mu$, whereas a few measure 126 $\mu \times 126\,\mu$ to 140 $\mu \times 140\,\mu$ (Plate VII, Fig. 5).

DISCUSSION

Rhizoctonia is known as the sterile mycelial condition of a basidiomycetous fungus allied to *Corticium* or *Hypochnus*. Both these fungi are soil dwellers. The unusually wide range of hosts attacked by *Rhizoctonia*, includes along with a number of conifers and cucurbits, jute, cotton, tobacco, citrus, strawberry, wheat, oat and several others. *R. solani* alone, has been reported on about 230 susceptible hosts belonging to 66 different families (Braun, 1930). The fungus has been described to produce a variety of symptoms on various hosts.

Among the species of *Kochia*, only *K. scoparia* L. is known to be affected from a few virus diseases and a damping-off caused by *Pythium debaryanum* (Erickson, 1947). Recently Bruehl (1951), while studying the nature of the crown rot disease in the wheat seedlings by *R. solani*, also tested a number of grass and non-grass hosts for their susceptibility to this pathogen. The list included *K. scoparia* as well, in which the infected plants were marked by

the general stunting and the brownish lesions at their collar region. This root-rot of *K. indica* Wight caused by *Rhizoctonia solani* Kuhn is, however, recorded for the first time.

Two fungi have been found associated with the diseased plants, of which *R. solani* has been proved to be pathogenic while the *Fusarium* sp. has been concluded to be a secondary fungus. The pathogen is able to infect through the roots and is unable to attack the hypocotyl. This is in contrast to its nature on potatoes, cotton and a few others, where the infection may be secured at the ground level also. The young *Kochia* seedlings succumb to infection within three days, become flaccid, lose turgidity and dry up within a week resembling the 'damping-off'. *R. solani* is also known to cause the 'damping-off' in seedlings in a wide number of plants. The older plants of *K. indica* react after three weeks of the inoculation. The general characters of the disease closely agree with those described by Samuel and Garrett (1932) for the root-rot of wheat and oats caused by *R. solani*. The decortication of the affected roots of *Kochia* is similar to that produced by the infection of *R. solani* through the first internode of corn seedlings (Ho and Melhus, 1940).

Rhizoctonia is known to be most active as a parasite at low temperatures and in nature the plants which are not killed usually recover from the infection at a little higher temperatures (Richards, 1921 and Walker, 1928). Also the temperature requirements for the different species of *Rhizoctonia* and also for the same species on different hosts vary greatly (Leclerg, 1939 and Samuel and Garrett, 1932). The optimum temperature for this strain of *R. solani* has been found to be 25° C. on potato dextrose agar and 30° C. on other media. Thus the one-month old *Kochia* seedlings are subjected to optimum growth temperature during the months of February and March and succumb readily, while the older plants survive for a longer period because of the higher range in temperature during May and June and therefore consequent slow growth and development of the pathogen in the host. The fact that diseased plants have been observed even in the late season, is suggestive of the fact that once the plants become infected earlier or later, they are unable to recover.

Summary

The paper deals with the root-rot of *Kochia indica* Wight caused by *Rhizoctonia solani* Kuhn which secures successful infection through the root of the host. The disease is described for the first time.

The external and internal symptomatology of the diseased plants has been described. The distribution of the fungus in different parts of the host has been studied in detail. In the seedling stage of the plant the disease symptoms resemble the 'damping-off'.

The morphology of the pathogen in culture and its temperature requirements have been determined.

ACKNOWLEDGMENT

The author is thankful to Professor S. N. Das-Gupta, D.SC. (Lond.), F.A.SC., F.N.I., for his keen interest and criticism during the course of this study.

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EXPLANATION OF PLATE VII

FIG. 1. (*a*) One-month old seedling showing the drooping of the shoot in the form of an arch.
(*b*) A healthy seedling of the same age.

FIG. 2. (*a*) Completely healthy plant which is short and erect with bushy foliage.
(*b*) Plant showing gradual withering of foliage. The branches towards the top are affected partially.
(*c*) Completely withered plant about a fortnight after the first visual symptom.

FIG. 3. Microphotograph of a portion of the central cylinder of the matured root to show the presence of sclerotia in the 'gap' while the hyphæ spread over the vessels on either side of the separated vascular rings, × 160.

FIG. 4. Microphotograph of the cross-section of the main central axis to show the radial distribution of the hyphæ over the vascular tissue, × 160.

FIG. 5. Microphotograph of the infected lateral branch showing the inter-cellular distribution of the hyphæ in the extrastelar tissue and the development of sclerotia towards the periphery. The hyphæ are thick, dark-brown and profusely branched, the branches arising at right angles, × 240.

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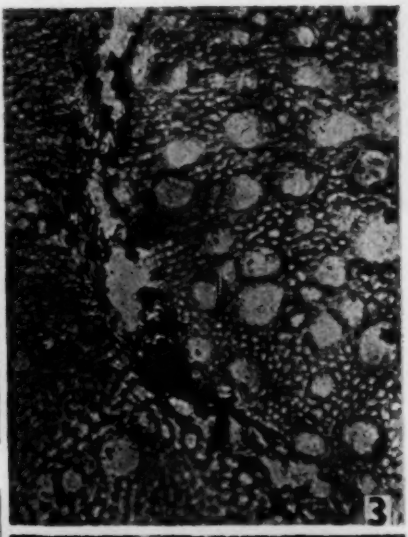

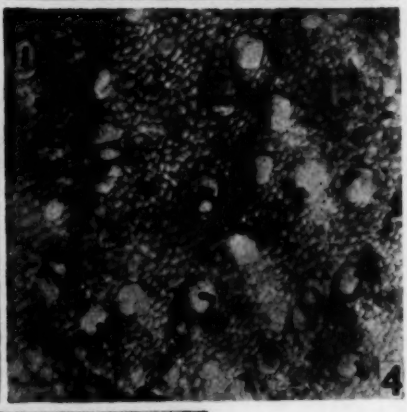

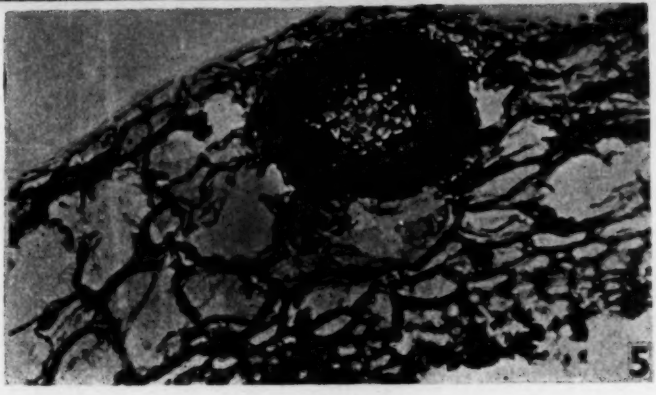

FIGS. 1–5

THE CHARACTERISTICS AND QUESTIONABLE TAXONOMIC POSITION OF THE OXALATE-DECOMPOSING BACTERIUM, *VIBRIO EXTORQUENS*

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In 1913, Bassalik isolated a rod-shaped organism from the excreta of an earthworm and from forest and garden soils and demonstrated that it was capable of utilizing oxalate as the sole carbon source. This organism which he called *Bacillus extorquens* represented the first record of an oxalate-decomposing microorganism. Janota in 1950 claimed to have isolated bacteria from polish soils which in every respect resembled the bacillus of Bassalik. She preferred to call this organism *Pseudomonas extorquens.* In the seventh and latest edition of *Bergey's Manual of Determinative Bacteriology* (Breed *et al.*, 1957) this bacterium has found a place in the genus *Vibrio*, the fortunes of its generic position having thus shifted over the last few decades from *Bacillus* to *Pseudomonas* and *Pseudomonas* to *Vibrio*. An important reason for the unstable taxonomic position of this bacterium appears to be the fact that Bassalik's original isolates had perished and that there was a dearth of other authentic strains on which to base descriptions. During the course of investigations on oxalate-decomposing bacteria, we were fortunate in encountering four strains which resembled the bacillus of Bassalik. The Polish strain called *Pseudomonas extorquens* was kindly made available to us by Dr. L. Janota of Warsaw University, Poland. Comparative and detailed studies on all the five strains were undertaken in our laboratories and they throw some reflections on the taxonomic position of Bassalik's bacillus—a position which in our opinion still remains questionable.

Experimental Methods

Strains.—Three of the five strains utilized in these investigations (07 *a*, GO_x 5 and O_x 8) were isolated from the intestines of the Indian earthworm

by the enrichment culture technique (Khambata and Bhat, 1957). A fourth strain ($N_2 5p$) was isolated from the digestive tract of the silkworm, *Bombyx mori*, L. (unpublished data). The fifth strain was Janota's *Pseudomonas extorquens* kindly sent to us by her from Poland. All five strains were maintained on nutrient agar and oxalate enrichment agar.

Morphological studies.—Apart from the conventional methods of studying bacterial morphology, morphological studies were also undertaken on slide preparations in which the growth of the bacterium could be followed *in situ*. A few drops of the culture suspension was placed on a clean sterile slide or coverslip in a sterile petri dish and allowed to dry in air. The dried area was then covered by a thin block of freshly poured and set nutrient agar. Preparations made in this manner were incubated at 28 to 30° C. for varying periods after which they were fixed (Bouin's fixative) and used for staining. Among the staining procedures employed, the tannic acid-crystal violet method of Robinow (1945) was found to be very useful in demonstrating the cell-wall. Flagella staining was performed by Loeffler's method, staining for metachromatic granules by Albert's method and staining for fatty inclusions by Sudan Black B.

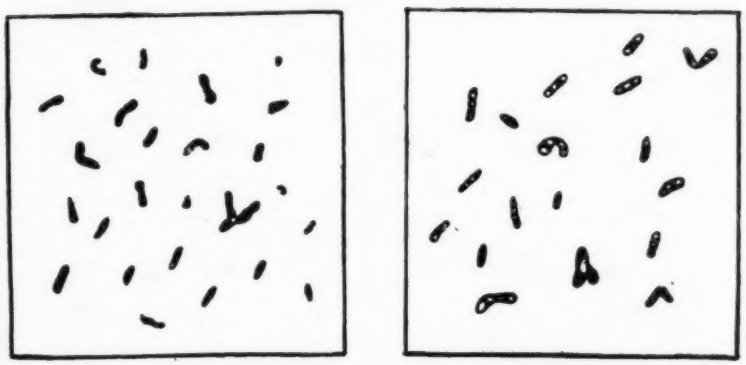
Physiological studies.—All conventional physiological tests were conducted, the methods followed being those recommended by the Society of American Bacteriologists in their *Manual of Microbiological Methods* (1957). In addition, an attempt was made to determine whether the carbohydrate metabolism of the organism was oxidative or fermentative by the relatively simple test recommended by Hugh and Leifson (1952). Penicillin sensitivity was determined both in broth cultures and by the agar cup method using *Micrococcus phyogenes* var. *aureus* strain P 209 as the control. Differences in sensitivity to antibiotics have been recommended by Shewan and Hodgkiss (1954) as a means of differentiating pseudomonads from other non-spore-forming rods.

Cross-agglutination studies with Arthrobacter *spp.*—The resemblance and apparent relationship of the five strains to species of *Arthrobacter* prompted experiments to determine whether an antigenic relationship exists between standard strains of *Arthrobacter* (kindly made available by Drs. G. B. Landerkin and Katznelson of the Canada Department of Agriculture) and the bacillus of Bassalik. Antisera (agglutinins) against three standard strains of *Arthrobacter*, *A. simplex*, *A. ureafaciens* and *A. pascens* were prepared in healthy rabbits with an immunization schedule of one intraperitoneal injection and two intravenous injections on consecutive days followed by a period of rest for the remaining four days of the week. This schedule was continued for

six weeks after which the animal was bled from the heart and the serum harvested and used for agglutination tests. Agglutination responses of all five of the test strains and the three homologous strains were determined by the microscopic slide test using serial dilutions of the respective sera. All other details of the test were as recommended by the Society of American Bacteriologists (1957).

Characterization

Morphological characteristics.—Straight to slightly bent rods of irregular size (2·0–2·7×1·0–1·3 microns), individual rods often varying in breadth along their length. Cells occurring singly, in pairs and occasionally in 'V', 'L', 'Y' and 'X' forms reminescent of the diphtheroids and the 'cystites' of *Arthrobacter* (Fig. 1 *a*). Gram negative in young culture but some cells show a distinct tendency to Gram positiveness particularly on aging. No septum revealed in cell-wall-stained preparations. Monochrome or Gram stained preparations presented a highly vacuolated appearance (Fig. 1 *b*). Two to three distinct denser areas were (usually polar) observable under phase contrast. These areas were also observed to be sudanophilic when stained with Sudan Black B. Metachromatic granules were not revealed by Albert's method. The cells were noncapsulated, non-sporeforming but motile. With the exception of strain O_x 8 which showed an additional lateral flagellum and $N_2 5p$ where no flagella could be demonstrated, the other three strains were found to possess a single polar flagellum.



a *b*

Fig. 1. Organisms stained lightly with dilute fuchsin to show vacuolated appearance.

Growth characteristics.—Colonies on nutrient agar were less than 1 mm. in size, smooth, round, entire, convex, opaque and pink to carrot red

in colour. Their internal structure was granular and compact. Colonies on Bhat and Barker's oxalate agar (1948) were similar to those on nutrient agar except for a slightly smaller size and the presence of a halo around each colony indicating oxalate decomposition. In nutrient broth and the liquid oxalate medium, growth was generally scant and characterized by a slight turbidity and a pink granular to membranous sediment. Growth on nutrient agar slants was not abundant. Moderately good growth was obtained on potato medium with the pigment varying from pink to coral red. Optimum temperature for growth was 27–30° C. with poorer growth at 37° C.

Physiological characteristics.—As a group the five strains were characterized by weak physiological activity. They failed to produce indole, hydrogen sulfide, amylase, gelatinase, cellulase or acetyl methyl carbinol. Likewise, none of the following carbohydrates were fermented in peptone water; xylose, glucose, maltose, lactose, sucrose, glycerol, mannitol, dextrin and starch. The methyl red test was also found to be negative. All five strains however producced catalase and a slight alkalinity in litmus milk. Ammonia was produced from nitrates by two strains (*GO$_x$* 5 and N$_2$5*p*) and one strain (07 *a*) produced nitrite only. In tests intended to determine the oxidative or fermentative nature of the organisms glucose and oxalate were used separately as carbon sources. With the sole exception of strains *O$_x$* 8 which was fermentative (anaerogenic) only towards oxalate, all other strains showed no acid production either under aerobic or anaerobic conditions. Penicillin sensitivity in broth cultures varied within the limits 0·2 units/ml. to 15·0 units/ml. Sensitivity determinations by the agar cup method was vitiated by the indifferent and poor growth of the organisms on the surface of nutrient agar.

Agglutination responses.—The results of the agglutination tests are presented in Table I. These show cross-agglutination between the *Arthrobacter* spp. and the strains under consideration in titres that are 10–30 times less than the homologous controls. All other necessary controls were also set during this test.

DISCUSSION

An examination of the five strains of Bassalik's bacillus reveals that despite a superficial resemblance to *Pseudomonas* and *Vibrio*, they share several characteristics with the soil diphtheroids, a group which has as yet been poorly studied and characterized. A classification of this group has however been attempted and in the latest edition of *Bergey's Manual of Determinative Bacteriology* (1957), the soil diphtheroids that do not decompose cellulose have been placed in the genus *Arthrobacter*. In Table II an

TABLE I

Agglutination responses of "Bassalik's bacillus" (P. extorquens) *with sera prepared against three* Arthrobacter *spp.*

Antiserum prepared against	Highest dilution of antiserum showing agglutination with							
	A. simplex	*A. ureafaciens*	*A. pascens*	07 *a*	*GO*, 5	*O*, 8	$N_2 5p$	*P. extorquens* (Janota)
A. simplex ..	1/50	1/8	1/8	1/2	1/2	1/2	1/2	1/4
A. ureafaciens ..	1/20	1/200	1/10	1/4	1/10	1/10	1/10	1/10
A. pascens ..	1/30	1/50	1/100	1/10	1/8	1/10	1/8	1/10

attempt has been made to compare some of the important features of Bassalik's bacillus with those that are generally accepted as characteristic of *Pseudomonas* and *Vibrio* on the one hand and *Arthrobacter* on the other. In its morphological details as well as in most of its physiological characteristics, the strains under consideration bear a close resemblance to *Arthrobacter*. Special mention must however be made of the marked vacuolation exhibited by the strains in monochrome-stained preparations and of the denser areas revealed in phase contrast studies which were found to be sudanophilic. The results of the agglutination tests indicate an antigenic relationship with *Arthrobacter*. Though penicillin sensitivity is comparable to that exhibited by *Arthrobacter*, it must be conceded that *Pseudomonas* as a group shows a wide variation in penicillin sensitivity.

It would appear that the Gram-negativity of the organism in young culture, its frequent curvature and its motility and predominantly polar flagellation, must have suggested affinities to *Vibrio* and *Pseudomonas*. However, it shares a great many other characteristics with *Arthrobacter* to which it appears to bear a closer resemblance. An examination of an appreciable number of pleomorphic soil bacteria in our laboratories has revealed that several of them are motile or sluggishly motile and bear polar flagella (Khambata and Bhat, 1955). Further, the ability to decompose oxalate, a characteristic which is a feature of these strains, has in the last decade been shown to exist in a variety of bacterial genera ranging from unbranched pseudomonads to branched streptomyces (Bhat and Khambata, 1955). It is therefore conceivable that the strains under consideration here may represent a phylogenic link between the pseudomonads and the diphtheroids. At any rate the

TABLE II

A comparison of some salient characteristics of "Bassalik's bacillus" (P. extorquens) *with those of* Pseudomonas, Vibrio *and* Arthrobacter

	Pseudomonas and *Vibrio*	*Arthrobacter*	"Bassalik's bacillus"
1.	Uniformly Gram negative at all stages of growth	Gram negative to Gram variable rods. Coccoid older cells Gram positive	Most strains Gram negative in young culture but with a tendency to Gram positivity on aging. Gram positive coccoidal elements in old cultures.
2.	Straight to curved rods that do not show angular arrangement, snapping division or 'cystites'	Swollen and club-shaped forms, frequently showing angular arrangement and snapping division	Curved to straight rods often swollen. Highly vacuolated. Tendency to angular arrangement. 'V', 'L' and 'X' shaped forms and cystites observed
3.	Generally motile with polar flagella. Lateral flagellation rare	Generally non-motile.* When motile, polar or lateral flagella reported	Motile with polar flagellum with the exception of one strain which shows lateral flagellation
4.	Grow well and rapidly on the surface of standard culture media or in liquid media	Growth soft and viscous on solid media. Generally not profuse in liquid media	Growth soft, viscous and not abundant on solid or liquid media
5.	Generally grow well at 37° C.	Little or no growth at 37° C.	Growth at 37° C. poorer than at 28° C.
6.	Indole production variable	Indole not produced	Indole not produced
7.	Glucose usually oxidised to acids and other intermediates and most species oxidative in their metabolism	Little or no acid from carbohydrates	Generally no acid production from carbohydrates under aerobic or anaerobic conditions. A single strain produces slight acid from oxalate under anaerobic conditions

* Studies in one of our laboratories on a large collection of bacteria having affinities with the *Arthrobacter* have revealed that several pleomorphic rods are motile and flagellated.

characterization of these five strains suggests the necessity for the examination of a larger collection of these and similar organisms and a reassessment of their taxonomic position.

Summary

A detailed account of the morphological, staining, penicillin sensitivity and serological peculiarities of five strains of an oxalate-decomposing bacterium including the well-recognized strain *Vibrio extorquens*, has been given. Inasmuch as all the strains share many of the characteristics of the genus *Arthrobacter* and not *Vibrio* the desirability of placing the bacterium in the former genus for the time being has been suggested. The possibility of the strains falling under an altogether new genus which represent a phylogenic link between the pseudomonads and diphtheroids has been speculated.

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NOTES ON SOME FUNGI FROM SOUTH INDIA—VIII

By T. S. Ramakrishnan, F.A.Sc.

(Pathologist, Rubber Board, Kottayam, S. India)

Received November 18, 1959

***Phytophthora palmivora* Butl.**

On *Artocarpus hirsuta* Lamk. causing severe defoliation, Kottayam (Kerala), 16–7–1959, T. S. R.

Artocarpus hirsuta is common in many of the residential compounds in Central Kerala. During the heavy rains in July a certain amount of defoliation has been observed in some trees growing in Kottayam District for the last three years. But in July 1959 several trees exhibited severe leaf-fall with over 50 to 80 per cent. of the leaves shed. The same type of defoliation was evident in some jack (*A. integrifolia* Lamk.) trees also. The increased severity this season is mainly due to the heavier rainfall (between 20 and 30 inches more than normal in June and July) received during 1959.

The leaves that are shed are either green or yellowish. Normally no lesions are seen on them. But in some, the basal portion of the midrib and the lamina on either side of this are affected and form a narrow dark-brown inverted V-shaped patch. However the petioles of the fallen leaves are always darkened and shrunk. The cortical portion of the twigs bearing the foliage exhibits considerable shrinkage and the presence of longitudinal splits. Sometimes the bark rots and sloughs off exposing the wood. Dieback of twigs is common.

Microscopic examination of the petiole and the twigs revealed that the tissues were permeated by non-septate hyaline hyphæ. These were mainly intercellular but the vessels in the vascular tissue had also been penetrated. When surface sterilised bits of the affected twigs or petioles were placed in Petri dishes containing sterilised water numerous sporangia of *Phytophthora* were produced in 24 hours. From these the fungus was brought into pure culture.

A woolly white aerial growth was formed on Quaker oats agar, filling the dishes and tubes in the course of a week. Numerous sporangia and chlamydospores were produced in a week. The sporangia were of the characteristic obpyriform shape, prominently papillate, terminal or intercalary and measuring 35–60 × 30–50 μ. The chlamydospores were more or less spherical,

intercalary and measured 40–60 μ in diameter. Even after two months oogonia or oospores were not observed in the cultures. The fungus was found to resemble some of the isolates of ***P. palmivora*** and was identified as a strain of this species.

Inoculation experiments were carried out with this isolate on young plants of *A. hirsuta*. The terminal young shoots and the petioles were readily infected in the course of a week and the leaves were shed. High humidity had to be maintained for successful infection. Positive infection of rubber shoots (*Hevea brasiliensis* Muell.) was also produced by this isolate. When tender shoots were inoculated typical symptoms of 'shoot rot' occurred causing rotting of the tender stem and the young leaves. The infection proceeded from the apex downwards to the lower storey of leaves. Rubber appears to be readily infected by more than one strain of *P. palmivora*. *A. hirsuta* is a new host for *Phytophthora* and defoliation of the trees is brought about by this pathogen just as in rubber. Young shoots of jack were also susceptible. The petiole was infected and the leaves were shed.

***Aecidium* sp.**

On leaves of *Tylophora asthmatica* Wight, Kottayam (Kerala), 25–5–1959, T. S. R.

Spots amphigenous, circular or irregular, yellow to yellowish-brown half to one inch in diameter; pycnia mainly epiphyllous, subepidermal, slightly protruding, flask-shaped, 90–120 × 60–80 μ; æcia hypophyllous, peridiate, 300–370 × 210–270 μ, peridial cells strongly verrucose on the inner surface; æciospores globose, minutely verruculose, catenulate, 30–40 × 25–31 μ, wall hyaline, sometimes irregularly thickened, contents yellowish orange.

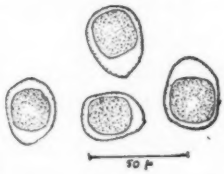

FIG. 1. Aeciospores of *Aecidium* sp.

Other stages of the rust were not present and hence it is placed in the form genus. A close resemblance to *A. nummulare* Berk. is noticed.

***Sclerotium rolfsii* Sacc.**

On *Citrus maxima* Merr. causing die-back of branches, Kottayam (Kerala), 10–7–1959, N. R. Pillay.

A whitish growth of the fungus covered the affected branches and twigs and caused the drying of the foliage and die-back of the branches during the rainy season in July 1959. On the surface of the branches embedded in the white growth were numerous round reddish-brown sclerotia of the fungus.

***Pellicularia filamentosa* (Pat.) Rogers**

On leaves of *Canna indica* L. causing blight, Pudukad (Kerala), 15–7–1959 T. S. R.

During the south-west monsoon rains in July 1959 a severe leaf blight occurred in a bed of Cannas in Pudukad destroying most of the foliage. To begin with, circular or irregular isolated or confluent lesions appear in different parts of the leaf-blade. These are dark-brown in the centre and surrounded by dull-green water-soaked margin about 4–5 mm. in width. In the course of four or five days the infection spreads and the entire leaf turns brown and is involved in a wet rot. When a diseased leaf is dried between sheets of blotting-paper some of the lesions appear as greyish-white patches against a dark-brown background.

The fungus mycelium can be detected especially on the lower surface of the lesions as branched whitish threads. Internally the hyphæ are mainly intercellular and hyaline. Sometimes the substomatal air-spaces contain aggregates of hyphæ. The entry into the tissues is through the stomata. Wherever the hyphal penetration had occurred the chlorophyllous tissue was turned brown in colour and the cells shrunk.

The pathogen was readily isolated from sufrace-sterilised bits of the affected leaves. On Quaker oats agar a rapid growth occurred with limited aerial mycelium. Many sclerotia developed in the course of a week. These were separate and 1–3 mm. in diameter or aggregated into bigger masses. The colour varied according to age, being initially cream but soon turning tawny brown. The surface was soft and velvetty.

Leaves of healthy *Canna* plants were inoculated with this isolate. In 48 hours lesions developed on all the inoculated leaves. In five more days entire leaves turned brown, the discolouration extending to the upper portion of the sheaths also. The spread of infection was rapid in rainy weather but was arrested when it became bright.

The foliage of young rubber (*Hevea brasiliensis*) plants were also readily infected. Lesions were formed in two days. In a week the leaflets were completely affected and some of them were shed. Others were held together by the mycelial growth between the contacting leaflets. The symptoms

produced were identical with those observed in the target spot disease of rubber caused by *P. filamentosa.*

This fungus has been recorded on several crop plants and weeds in Kerala. Ramakrishnan K. and T. S. (1949) obtained artificial infection of *Canna* leaves on inoculation with an isolate of *P. filamentosa* from *Maranta arundinacea* L. Now natural infection of *Canna* has been observed. The present isolate of the pathogen exhibits some cultural variations from the isolate on *Maranta.* It appears that many strains of this species occur in Kerala. They are not however specialised in their parasitism and readily pass on from one host to another. The high rainfall during the south-west monsoon period is apparently favourable for their development and spread.

***Corynespora cassiicola* (Berk. and Curt.) Wei**

Wei, C. T. (1950), "Notes on *Corynespora cassiicola,*" Mycological Papers, 34, Commonw. Myc. Inst. Kew.

On leaves of *Hevea brasiliensis* Muell., Kottayam (Kerala), 10-12-1958.

Leaf spots circular to irregular, varying in size from 1–6 mm. in diameter, amphigenous, centre brown to whitish, with a dark-brown margin, often with a yellowish halo round the spot, sometimes having a shot-hole appearance; conidiophores emerging singly or in small tufts or produced on aerial mycelium on the surface of the sopt, brown, septate, variable in size,

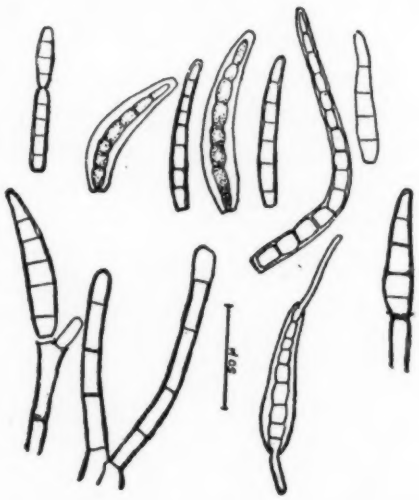

FIG. 2. Conidiophores and conidia of *Corynespora cassiicola*

85–300 × 6–10 μ, proliferating through the terminal scar or through injured ends; conidia borne singly or in chains, connected with the conidiophore by a hyaline isthmus, obclavate or cylindrical, multiseptate with 2–14 septa, straight or often slightly curved, tapering towards the apex, pale-brown, often with a thick wall, prominent hilum, 40–210×8–18 μ.

This disease has a superficial resemblance to 'birds-eye spot' disease of rubber caused by *Helminthosporium heveae* Petch. But the persistent halo and the earlier change in the colour of the spot to white help to distinguish this from the other. Further the malformation and crinkling of the tender leaves which are sometimes noticed in plants infected by *H. heveae* have not been observed in this case. However microscopic examination will be the decisive method of diagnosis. This disease is prevalent more during the drier months and sometimes causes defoliation.

The fungus was isolated by single spore method. On Quaker oats agar a luxuriant greyish aerial growth is formed. The substratum is coloured deep pinkish violet. A grey aerial growth develops on sterilised rubber leaves. Conidia and conidiophores are produced in cultures also. Symptoms of infection become evident five to six days after inoculation. Tender leaflets are infected in three to four days. On mature leaflets the incubation period may sometimes extend to nine days.

Entrance is effected through either surface of the leaflets.

The fungus closely resembles *Helminthosporium*. Among its synonyms are more than one species of *Helminthosporium*. However both in morphological and cultural characters this fungus differs from *H. heveae*.

I am indebted to Dr. R. S. Vasudeva, Head of the Division of Mycology, I.A.R.I., New Delhi, for kindly arranging for the despatch of specimens and slides to the Commonwealth Mycological Institute, Kew, for the identification of *Corynespora*.

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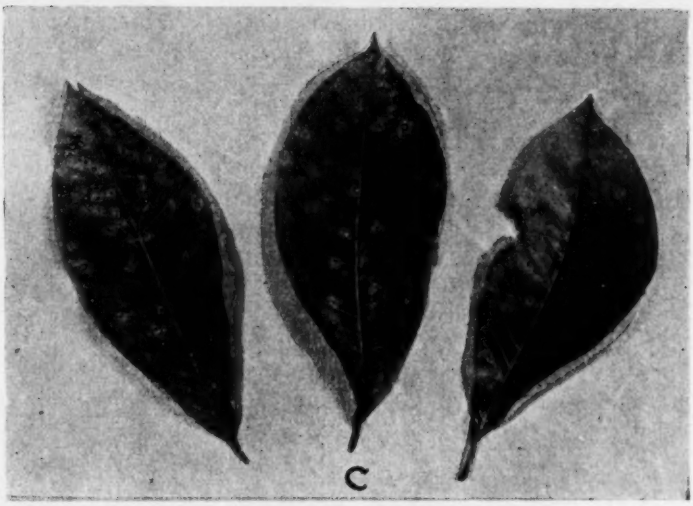

A. Photograph of *Artocarpus hirsuta* showing defoliation.
B. A branch of *Citrus maxima* with *Sclerotium rolfsii* growing on it and with a few sclerotia.
C. Leaflets of *Hevea brasiliensis* with leafspots caused by *Corynespora cassiicola*.

ON A COLLECTION OF MACROLICHENS BY THE INDIAN EXPEDITION TO CHO-OYU, EAST NEPAL

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Received September 1, 1959

(Communicated by Dr. S. N. Das Gupta, F.A.Sc.)

The Indian mountaineering expedition undertaken in 1958, to the world's sixth highest peak—Cho-Oyu, in East Nepal, provided excellent opportunities to simultaneously explore scientific facts particularly on Natural Sciences from the regions visited. To get all possible data of botanical interest and collect plants, the Government of India deputed a senior member of Botanical Survey of India to accompany the expedition. Due care seems to have been taken to the collection of all groups of plants with the result that lichens are also well represented in the collection. The macrolichens (foliose and fruticose types) alone, which have been sent to me for determination, for the present, constituted 45 packets. Except in few cases (when it becomes almost unavoidable in bulk collection), there was no mixture of different lichens in the packets. The specimens have been collected from the altitudes of about 1880 m. (= 6,200 ft.) to 5455 m. (= 17,000 ft.), which corresponds to the temperate and alpine zones of the Himalayas, where lichens abound in variety and abundance constituting an important component of the vegetation. The variety is well reflected in the occurrence of 38 species in a collection of 45 packets.

All these specimens were collected by Mr. Rolla Seshagiri Rao, the Regional Botanist, Botanical Survey of India, who accompanied the expedition, and the numbers of the specimens pertain to his field numbers. I am thankful to him for placing at my disposal this excellent collection of lichens for determination and also for allowing me to retain parts from all the specimens, with the field notes on the labels, for my herbarium. The other set is preserved at the Shillong Herbarium of the Botanical Survey of India.

I also take the opportunity of expressing my thanks to Professor S. N. Das-Gupta for constant encouragement and helpful suggestions and to Mr. M. B. Raizada for the loan of the part of the Isotype of *Cetraria ambigua* from Dehra Dun Herbarium.

The species as determined from the collection are arranged below according to Zahlbruckner's system.

STICTACEÆ

***Lobaria isidiosa* (Müll. Arg.) Vain.**

Common on bark of *Rhododendron campanulata*, near Debuche, alt. 3657–3962 m., April 23, 1958 (No. 13834).

Thallus foliose, characteristic, widely lobate laciniate, darkish-brown, isidiose along the margins and on the ridges of the scrobiculate surface. Medulla K_, Cl_, Pd_. Sterile.

Common in temperate Himalayas, in shade.

***Sticta henryana* Müll. Arg.**

On bark of *Rhododendron campanulata*, near Debuche, alt. 3657–3962 m., April 23, 1958 (No. 13833/A).

Thallus foliose, sinuate lobate, lobes up to 1 cm. broad, smooth, ashy-grey to pale-greyish; underside pale with round cyphellæ, dark-brown rhizinæ and long-branched rhizinose fibrils. Medulla K_, Cl_, K (Cl)_, Pd_. Sterile.

The species is a new report for this area as it has so far been known only from China.

***Sticta platyphylloides* Nyl.**

Mixed with the last one (*S. henryana*) in the same packet, separated out and numbered as 13833/B.

Thallus foliose, sinuate lobate, glaucous pale-grey to brownish, subpruinose under lens on the upper surface; underside with cyphellæ and pale grey tomentum of rhizinæ. Medulla K_, Cl_, K (Cl)_, Pd_. Apothecia profuse; subpodicellate, up to 6 mm. in diameter; disc plane to convex, red-brown; margin minutely crenate; spores elongate, fusiform, 40–50 × 6 μ in size.

It is a new report for this region, being known so far, like the previous species from China only.

CLADONIACEÆ

***Cladonia mitis* Sandst.**

Along rocky crevices, Chule to Lhenjo, alt. 4724 m., May 11, 1958 (No. 13899). Common on soil, Rhododendron and grass-covered soil, along Relmo Lak area, alt. 4998 m., May 12, 1958 (No. 13916).

Thallus densely bushy, podetia pale-white to pale greyish, subarachnoid tomentose and semipellucid, usually terminating in 2–3 branchlets at the apex. Podetia K_, Pd_. Sterile.

***Cladonia sylvatica* (L.) Harm.**

On soil, slopes below Minjbo, alt. 4480 m., April 20, 1958 (No. 13759).

Thallus bushy, subsimilar to the last species (*Cl. mitis*) but distinguished by the stouter podetia which are characteristically multiple branched, conidia colourless. Podetia K-, Pd + orange-red to red. Sterile.

***Cladonia aggregata* (Sw.) Ach.**

Common on tree branches, Cho-Oyu, near Nigala, alt. 2133 m., March 20, 1958 (No. 13558). Thallus bushy, up to 4 cm. tall. Podetia densely congested and characteristically elliptically perforated at intervals, light-pale to light-chestnut coloured.

***Cladonia pleurota* (Flk.) Schaer.**

On soil, Lobuje to Pangboche, alt. 4267 m., April 26, 1958 (No. 13820). Near Debuche, alt. 3657 m., April 23, 1958 (No. 13836).

Primary thallus squamulose, podetia smooth elongated and gradually widened to a short scyphus, Pd-. The first specimen (No. 13820) is fertile and latter (No. 13836) sterile.

***Cladonia pyxidata* (L.) Fr.**

On soil-covered rock, Dole, alt. 4267 m., May 15, 1958 (No. 13937).

Primary thallus squamulose, podetia short with wide funnel-shaped scyphus, surface scurvy squamulose, Pd + orange-red. Sterile.

***Cladonia* cf. *ceratophyllina* (Nyl.) Vain.**

On soil, near Debuche, alt. 3657 m., April 28, 1958 (No. 13838).

Specimen with few podetia which are tapering and unilaterally squamulose, Pd + orange-red.

***Cladonia* cf. *verticillata* Hoffm.**

On soil, near Debuche, alt. 3657 m., April 28, 1958 (No. 13837).

Podetia linear elongated with scyphus at the top, grey-brown to brownish, Pd + orange-red. Fertile.

***Stereocaulon myriocarpum* Th. Fr. var. *orizabæ* Th. Fr.**

On rocks, near Gora shop, alt. 5303 m., April 26, 1958 (No. 13804).

Thallus erect bushy and congested, up to 4·5 cm. tall. Pseudopodetia branched from the base, much entangled, densely covered by the characteristic ashy grey phylloclades. Apothecia copious, terminal or lateral, small, rounded and brown; spores 30–40×4–9 μ in size. Specimen No. 13804 fertile and No. 13938 sterile.

***Stereocaulon piluliferum* Th. Fr.**

On dry rocks, Ghat to Namchebazar, alt. 2743 m., April 11, 1958 (No. 13727).

Pseudopodetia erect, up to 3 cm. tall, scarcely branched or branches few, phylloclades well developed, linear, tapering, cephalodia sacculate type. Apothecia terminal, convex, dark-brown; spores elongate, fusiform, multicelled, 100–140×3–5 μ in size.

Endemic in the temperate Himalayas. Usually growing gregariously on moist exposed rocks.

PARMELIACEÆ

***Parmelia vittata* (Ach.) Röhl.**

On Rosa shrubs, Thengbock to Pangbock, alt. 3992 m., April 19, 1958 (No. 13740/C).

Only few fragments; the laciniæ with the characteristic, apically leprose soredia. As mixture with *Parmelia cirrhata*, etc.

***Parmelia cirrhata* Fr.**

On shrubs near Mahabir village, alt. 2438–2682 m., April 4, 1958 (No. 13638). On Rosa shrubs, Thengbock to Panbock, alt. 3992 m. April 19, 1958 (No. 13740/A)—Specimens in both somewhat typical though in the latter the marginal rhizinæ are much longer.

On trees and shrubs near Nigala village, alt. 2133 m., March 29, 1958 (No. 13561)—laciniæ narrow, apothecia almost subterminal, large and lobulate.

On twigs near Debuche, alt. 3657–3962 m., April 28, 1958 (No. 13835)—mixture of typical *cirrhata* and another with *cirrhata* like thallus with the difference that the laciniæ are about 3 mm. broad at base and gradually taper to 0·5 mm. apically. Underside black with stiff rhizinæ.

P. cirrhata is the most abundant and widely distributed species in the temperate Himalayas from East to West. Distributed in many other temperate parts of the world.

***Parmelia stenophylla* (Ach.) DR.**

On rocks, Lobuje to Pangboche, alt. 4572 m., April 26, 1958 (No. 13822). At Kisan Yak grazing village, alt. 4480 m., May 5, 1958 (No. 13868).

Thallus foliose pale-yellow, laciniæ much imbricated, usually 0·5–1 mm. broad, rarely narrower. Underside with pale-grey rhizinæ. Occasionally laciniæ with characteristically broadened lobes. Medulla K + red, Cl-, Pd + deep-yellow to orange. Chiefly a European species, extending to the Himalayas. The laciniæ of the European specimens are broader; even the laciniæ in specimens from Kashmir (preserved in my herbarium) are broader than in these Nepal specimens.

***Parmelia reticulata* Tayl.**

On twigs of Berberis, near Nigala village, alt. 2133 m., March 29, 1958 (No. 13557).

A small piece but easily distinguished by the minute, white reticulations on the upper surface (distinct under lens) and the globose marginal soralia which are often confluent. Medulla K + yellow changing to deep blood-red.

***Parmelia nimandairana* Zahlbr.**

On branches of *Rhododendron arboreum*, near Nigala village, alt. 1889 m., March 29, 1958 (No. 13563).

Thallus foliose, sinuate lobate, yellowish-grey to grey, densely isidiose towards the central part, underside with black rhizinæ up to the margin. Medulla K + red, Pd + deep yellow to orange.

***Parmelia caperata* (L.) Ach. var. *cylisphora* Ach.**

On rock, Thyangbock from Namchebazar, alt. 2743 m., April 16, 1958 (No. 13726).

Thallus adpressed, foliose, pale to pale-grey, centrally ashy-grey, lobes imbricated and convoluted, soredia superficial on the raised submarginal areas and granular. Medulla K–, Cl–, Pd + red.

***Parmelia* cf. *cetrarioides* Del.**

On Rhododendron, near Debuche, alt. 3657, April 28, 1958 (No. 13833/C) mixed with the *Sticta* species described.

Thallus foliose imbricated, lobes marginally sorediate and undulate. Upper side pale glaucous grey, smooth with dispersed minute pseudocyphellæ, which are also present on the chestnut-brown to black underside;— scattered rhizinæ in the central part only. Medulla. K_, Cl_, Pd_.

***Parmelia tinctorum* Despr.**

Abundant on rocks, Dolaghat to Bhumlutar, alt. 1981 m., March 29, 1958 (No. 13537).

Thallus glaucous grey to ashy-grey, adpressed, densely isidiate in the central part and smooth in the peripheral parts of the lobes. Medulla K_, Cl + red, Pd_. Easily distinguished by the colour of thallus, isidiate nature combined by the positive Cl_ reaction.

A widely distributed species.

***Parmelia nilgherrensis* Nyl.**

Fairly abundant on twigs, near Mahabir village, alt. 2438 m., April 4, 1958 (No. 13637).

Thallus foliose, thick, coriaceous, glaucous pale-grey, laciniate lobate and much imbricated, margin black ciliate. No. soredia and isidia. Medulla K_, Cl_. Apothecia large subpodicellate, spores 20–22 × 12–13 μ in size.

***Cetraria everniella* (Nyl.) Krmphbr.**

(Habitat not given), slopes below Minjbo, alt. 4450 m., April 20, 1958 (No. 13760). Lobuje to Pangboche, alt. 4572 m., April 26, 1958 (No. 13824).

Thallus fruticose, erect bushy; laciniæ dorsiventral, narrow, dichotomously branched and congested; smooth, glaucous pale above and lacunose pale on underside. The laciniæ in No. 13760 are narrower to sometimes subterete, underside more prominently lacunose. Medulla croceus (sometimes it is colourless in young parts).

Thallus superficially Pd + red to red-brown after a long interval (10–12 hours) on drying. Medulla K_, Cl_, Pd_. Sterile.

Cetraria* cf. *everniella

On rock and soil, Chukpula to Lobuje, alt. 4998 m., April 22, 1958 (No. 13786).

Thallus erect to 4 cm. tall, bushy, fronds subdichotomously divided and congested, subcylindrical to cylindrical, 1–2 mm. wide, irregularly lightly

lacunose; minute white tubercles on ridges of lacinæ, singly or in groups, often associated with or in the neighbourhood of black pycnidial fibrils, fronds spongy to touch or pressure (not so in *everniella*). Algal zone on all sides or in the greater part of the circumference in the subcortical region. Medulla croceus, K_, Cl_, Pd_. Thallus surface externally Pd + red to red-brown after a long interval as stated above in *everniella*. Sterile.

It compares favourably to *C. everniella* in general characters but differs in the subcylindrical to cylindrical nature of fronds which are spongy to pressure, presence of algal zone in a greater part on all sides and in the absence of differentiation into dorsiventral nature except sometimes in the apical parts. Thus it may be considered either a variety or only an ecological variation.

***Cetraria* cf. *ambigua* Bab.**

On rock, soil and Rhododendron, Pambochi to Mingbo, alt. 4480 m., April 20, 1958 (No. 13758).

Thallus suberect to erect, narrowly lobate laciniate, laciniæ linear, 2–4 mm. broad, divaricately divided and imbricated, margin undulated and often recurved on the upper side thereby making them (laciniæ) semicanaliculate. Upper side smooth straw pale, lower side minutely lacunose (lacunæ more prominent in older parts) and pale. Margin beset with minute cylindrical brown fibrils bearing apical black pycnidia. Medulla white. Thallus externally (surface) Pd + red to brownish-red after long interval (comparable to *everniella*). Medulla K_, Cl_, Pd_.

Apothecia marginally terminal, rounded, up to 3 mm. in diameter, reticulately lacunose on underside, disc concave, brown, smooth, margin entire to minutely crenate. Asci immature.

The specimen compares favourably with the description of *Platysma ambiguum* (syn. of *Cetraria ambigua* Bab.) by Nylander (1860, p. 344). However, it differs from the Isotype of the species, preserved in the Dehra Dun Herbarium, in being somewhat thinner in texture, in the straw pale to pale in colour and the non-palmate branching of the laciniæ. The Isotype piece is palmately branched; smooth, brown on upper side and prominently lacunose up to the apex, and brown on lower side (the age of specimen may partly be responsible for the browning as I have noticed the specimens of *C. everniella* turned light-brownish collected just 9 years ago).

USNEACEÆ

***Letharia vulpina* (L.) Hue.**

On trees and shrubs, Lobuje to Pangboche, alt. 4572 m., April 26, 1958 (No. 13823). On Juniperus, Porcha to Namchebazar, alt. 3962 m., April 30, 1958 (No. 13848).

Thallus fruticose, light greyish-pale to pale, laciniæ not dorsiventral, subcylindrical, dichotomously branched and attenuating apically, tip with a brownish point, surface angularly lacunose, densely isidiose and furfuraceous. Medulla white of loose hyphæ, K_, Pd_. Sterile.

Though well distributed in the mountains of the Northern Hemisphere, a review of the literature reveals that it has not been reported so far from the Himalayas and thereby forms a new report for this area.

***Alectoria sulcata* (Lév.) Nyl.**

On Quercus trees, Shete to Jambosi, alt. 2971 m., April 6, 1958 (No. 13679).

Thallus fruticose, laciniæ filamentous, subcylindrical, divaricately branched and attenuated apically, smooth, ± furrowed on one side, setaceous pale-grey to olive in colour. Apothecia terminal, up to about 6 mm. in diameter, disc brownish, pruinose; margin thin, spores colourless, oval ellipsoid, single-celled, 36–44 × 12–16 μ in size.

***Alectoria* spp.**

On branches of bushy Rhododendron, Thaparma to Pheriche, alt. 4511 m., April 21, 1958 (No. 13768).

Thallus fruticose filamentous, densely branched and branches attenuated and entangled, dark-brown to almost black, light brownish toward apex. In the packet there are two types well entangled, subsimilar in thallus characters except that one is with white maculate soralia and other esorediate.

***Ramalina sinensis* Jatta.**

On small twigs, Lobuje to Pangboche, alt. 4267 m., April 26, 1958 (No. 13819).

Thallus fruticose, small, broad, deeply laciniate, longitudinally venose, light glaucous pale to brownish. No soredia. Apothecia copious, terminal, up to 5 mm. in diameter, spores 12–15 × 6–7 μ in size, straight or curved. Thallus and medulla K_, Pd_.

***Ramalina subcomplanata* Nyl.**

On moss covered soil, near Nigala village, alt. 2133 m., March 29, 1958 (No. 13560/A).

Thallus fruticose divaricately branched; laciniæ narrow, flattened or ± strap-shaped, brownish in basal parts and pale glaucous grey upwards. Surface lacunose nervose with white pseudocyphellæ. Medulla white, K–, Pd–. Apothecia copious, spores 11–16×5–7 μ in size.

Though endemic yet well distributed and abundant in the temperate Himalayas.

***Ramalina conduplicans* Vain.**

Associated as a mixture with the former (*R. subcomplanata*) and separated as No. 13560/B.

Thallus subsimilar to *subcomplanata* but medulla in *conduplicans* K + red, Pd + red or reddish.

A species originally reported from Siam forms a new report for the Himalayas.

***Usnea Hookeri* Mot.**

On dry soil, slopes above Gora Shop, alt. 5425 m., April 25, 1958 (No. 13792). Chule to Lhengo, alt. 4724 m., May 11, 1958 (No. 13900).

Thallus consisting of an irregularly flattened base from which arise the suberect to erect fronds branching divaricately; branches divergent, flexuose, intricated and attenuated apically. Basally fronds whitish to dark-brown or black and the upper half deep-orange or saffron-yellow, surface longitudinally finely reticularly rugulose. Orange part of thallus K + deep purple violet.

The species is endemic in the Himalayas and Tibet. It is reported to be used for dyeing wool in Tibet, thereby giving indication of its abundance in that country, though in the Himalayas it is of rare occurrence.

***Usnea flexilis* Strtn.**

Near Mahabir village, alt. 2438–2682 m., April 4, 1958 (No. 13634).

Thallus pendulous, filamentous, dichotomously branched, articulated and constricted at joints, flaccid in between the joints, and gradually attenuated apically. Surface straw pale, smooth with minute white pseudocyphellæ. Medulla white, K + red. Sterile.

***Usnea pectinata* Tayl.**

Abundant on branches of Betula and Rhododendron, Thengboche, alt. 3962 m., April 29, 1958 (No. 13844).

Thallus pendulous of long filaments, which are uniformly thin (0·2-0·3 mm.), lateral branches at right angles to the main axes, pale glaucous grey in colour. Central chondroid axis of filaments I_. Apothecia few, on short lateral branches, up to 1·5 mm. in diameter, disc convex, carneous pale to pale, subpruinose, with a thin whitish margin and few to many marginal cilia. Spores oval, single-celled, colourless, 9–10 × 5–6 μ in size. Subsimilar to *U. longissima* but distinguished by the negative I-reaction in central axis (in *U. longissima* axis I + blue).

***Usnea Hossei* Vain.**

On Quercus trees, Shete to Jambosi, alt. 2971 m., April 6, 1958 (No. 13676/A).

Thallus pendulous, filamentous, main branches irregularly thickish (0·5–1 mm.), dark-brown or glaucous grey-brown, lateral branches divergent pale towards the apex. Cortex persistent in the main filaments, central chondroid axis light yellowish I_. Medulla K_.

A Malayan species showing the extension of its distribution to the Himalayas. It has been collected from East Nepal by me earlier (in 1953) as well. The report is being made for the first time now from this area for the species.

***Usnea thomsonii* Strtn. subsp. *arborea* (Strtn.) Mot.**

Mixed with the last species (*U. hossei*) and separated as No. 13676/B.

Thallus fruticose, sympodially branched, somewhat inflated in the middle parts, pale greenish grey. Surface waxy, distinctly papillate and with dispersed white maculæ (= soralia) of irregular shape. Medulla arachnoid, K + gradually red. Apothecia copiously present, margin ciliate.

Endemic in the Himalayas.

A sterile specimen (No. 13739) from Thengboche, alt. 3992, April 16, 1958 is also comparable to this species.

***Thamnolia vermicularis* (Sw.) Ach.**

On soil, in slopes above Gora Shop, alt. 5455 m., April 25, 1958 No. 13793).

Thallus erect of simple or sparingly branched apically tapering, smooth fronds of bone-white colour. Thallus Pd + yellow then later presistently orange-red. Easily recognised by its form which resembles to small white worms.

It is reported "used as offering while praying in the monasteries" (R. S. Rao).

PHYSCIACEÆ

***Anaptychia esorediata* (Vain.) DR. *et* Lynge.**

On Quercus trunk, Shete to Jambosi, alt. 2971 m., April 6, 1958 (No. 13680).

***Anaptychia leucomelæna* var. *angustifolia* (Mey. *et* Flot.) Müll. Arg.**

On soil growing along with mosses, also mixed with other lichens, near Nigala village, alt. 2133 m., March 29, 1958 (No. 13559). Thangboche to Pangboche along with *Parmelia cirrhata* No. (13740/D).

Thallus pendulous, laciniæ linear, narrow. Sterile.

***Anaptychia leucomelæna* var. *sorediosa* Jatta**

Over stones, Dole to Namchebazar, alt. 4084 m., May 16, 1958 (No. 13959).

Thallus pendulous, laciniæ linear, copiously sorediate on the upper side and along the margins. Sterile.

***Anaptychia pellucida* Awasthi**

On Rosa twig, Thengboche to Pangbock, alt. 3992 m., April 19, 1958 (No. 13740 E).—Mixed with *Parmelia cirrhata*, etc.

Thallus pendulous, fruticose ashy grey. Apothecia epruinose with thin margin, spores distinctively large (60–70 μ long), 2-celled, brown and pellucid.

The specimens are somewhat smaller than the type (Awasthi, 1957) but it is easily distinguished from its nearest ally *A. podocarpa* by the epruinose apothecia, larger and pellucid spores.

Only a few years back, Asahina (1955) described 62 species under 19 genera of lichens from the collection made by the Japanese Expeditionists to the Nepal Himalayas. The present collection though smaller in the number

of species (38 spp. under 12 genera) yet includes the 14 species as new additions to that list of Nepal lichens, which are *Sticta henryana, St. platyphylloides, Cladonia mitis, Parmelia stenophylla, P. reticulata, Letharia vulpina, Alectoria sulcata, Ramalina subcomplanata, R. conduplicans, Usnea Hookeri, U. pectinata, U. Hossei* and *Anaptychia pellucida.*

SUMMARY

The paper deals with 38 species under 12 genera of lichens that had been collected by Mr. R. S. Rao, Botanist Member of the Indian Expedition to Cho-Oyu in East Nepal. Five species as new reports for the Himalayas are described.

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THE GENITALIA OF *ORYCTES RHINOCEROS* LINN. (COLEOPTERA, LAMELLICORNIA, DYNASTINÆ)

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I. Introduction

There is a growing feeling among students of taxonomy that the importance of reproductive organs has been too long ignored in the systematic works of insects. The lack of understanding of the fundamental nature of male genital organs by morphologists has resulted in nomenclatural confusion. This has been a stumbling block in the progress of work. Although the conclusions of the morphologists are not entirely in agreement, much has been

done towards the understanding of the subject. A number of publications have appeared on the morphology and embryology of the genital organs of Coleoptera. Most of them are by foreign authors, conspicuous among them are Sharp and Muir (1912), Tanner (1927) and Metcalf (1932). Among the Indian workers Pruthi (1912), Mukerji and Bhuya (1935), Pradhan (1935), Rakshpal (1946 and 1947) and Srivastava (1935 *a*, 1935 *b* and 1954) have contributed to the knowledge of genitalia in Coleoptera. We are still ignorant about the reproductive organs of most of our common beetles and hence the need for further studies. With this view the present authors studied the genitalia of *Oryctes rhinoceros* Linn. in detail. The economic importance of this insect drew our attention. These insects cause considerable loss to the cultivators of palms. They feed on soft tissue of the growing apices of the palms. Frequently the growth is arrested and the palm whithers away unless it is checked in time. A detailed knowledge of the reproductive system, no doubt, will help to determine measures against the multiplication of these harmful insects.

Lefroy (1909) placed *Oryctes rhinoceros* in the subfamily Dynastinæ. It is a large beetle with a thick muscular body and pale black in colour. Their larvæ are generally seen around the cattle-sheds and in heaps of decaying organic matter. The head capsule of the adult insect displays sexual dimorphism; the male possessing the frontal horn while the female has carina.

II. Material and Method

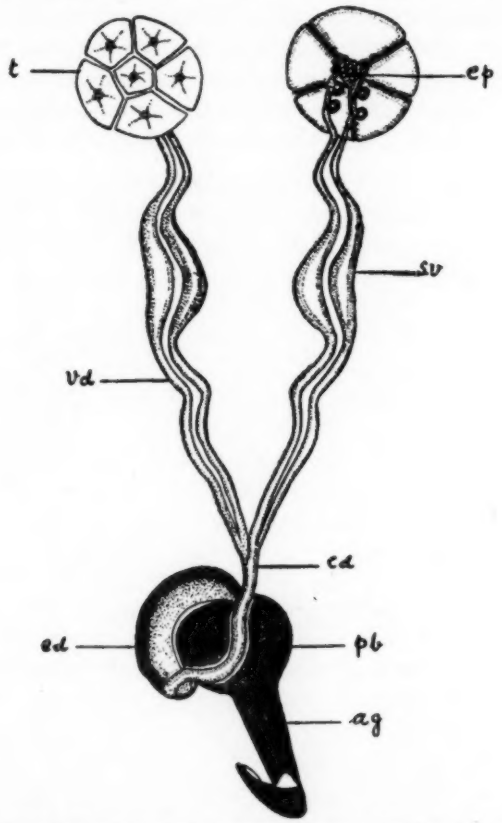
The insects were procured from Kerala State and were preserved in F.A.A. (formalin, acetic acid and alcohol) solution. A number of them were dissected under stereoscopic binocular microscope. Complete sketches were drawn of the internal genitalia in relation to the neighbouring organs. For the study of the external genitalia the insects were treated in 10% potassium hydroxide solution to make them transparent.

III. The Male Genitalia

(*a*) *Internal Genitalia*

The testes are two in number situated one on either side. It is interesting to note that the position of the two testes, is not in the same plane. The right testis is situated at the level of the sixth abdominal sternite; its anterior part is slightly projected in the distal part of the fifth abdominal sternite, while the posterior portion reaches even up to the pygidium. The right testis (4 to 6 mm. in diameter) consists of six follicles; one occupying the central position and the remaining five arranged around the former to give

the testis, a rosette form. The central follicle is the largest one. Three follicles surrounding the proximal border of the central one, are also large and pentagonal. The remaining two, which form the distal part of the testis, are small and circular in appearance. Each follicle consists of six to eight lobes; one lobe occupying the central position and the rest surrounding the former. The central lobe of the follicle is invariably found to be smallest in size. Due to the circular arrangement of the lobes a depression is formed in the centre of each follicle on the dorsal side. The ventral side of the testis presents a cup-shaped appearance, the middle portion of which is filled with a thick assemblage of fine tubules, the vasa efferentia. Curiously enough, each testicular follicle also has a central depression on the ventral side. The dorsal and ventral depressions of the follicle correspond in their positions.



TEXT-FIG. 1. The male genitalia of *Oryctes rhinoceros* showing the dorsal and ventral view of the left and right testes respectively.

The left testis is smaller in size than the right one, measuring from 3 to 5 mm. in diameter. It extends from the middle of the fifth abdominal sternite to the pygidium. The follicles of the left testis are similar in their shape, number and consistency to those of its counterpart of the right side. However, the arrangement of the follicles of the left testis is not a perfect rosette form.

Most of the workers have not dealt with the description of the vasa efferentia. Mukerji and Bhuya (1937), Williams (1945), Rakshpal (1947) and Dutt (1958) omitted it. In *Oryctes rhinoceros* from each follicle of the testis, a number of very fine ductules arise and converge in the ventral cup-shaped area. The follicles are so closely applied to each other that it is not possible to ascertain the exact number of ductules coming out of them. In the central portion of the ventral cup-shaped area the ductules form two thread-like ducts. These two ducts are the vasa efferentia; they coil around each other and form the epididymus (Snodgrass, 1935). It is from the epididymus that two fine tubes, the vasa deferentia arise and run posteriorly. Thus four vasa deferentia are present, two arising from each testis.

Each vas deferens is divisible into three parts; a proximal thread-like part; a middle swollen part and a distal narrow part. The entire length of the vas deferens varies from 20 to 25 mm. The proximal part of the vas deferens arises from the epididymus as a fine coiled tube which traverses posteriorly, like a watch-spring of about 6 mm. It gradually widens in its course and enlarges ultimately into a middle chamber, the seminal vesicle, which is oblong in shape. According to Snodgrass (1935) and Dutt (1958) the seminal vesicle is gradually formed at the distal end of the vas deferens. In *Bruchus quadrimaculatus* (Mukerji and Bhuya, 1937) the seminal vesicle is formed in the middle of the vas deferens, a condition similar to that of *Oryctes rhinoceros*. The vesicular seminalis is the widest portion of the vas deferens. The two vasa deferentia of each testis coil around each other so closely to appear as if formed of a single duct. Due to the coiling of the duct the vesicula seminalis of each vas deferens is convoluted. Distal to the vesicula seminalis, the duct suddenly narrows to form the third part of the vas deferens. The narrow duct is about 10 mm. in length and is of uniform calibre throughout. The four vasa deferentia unite to form a common duct, the ejaculatory duct, which is situated slightly to the right side in the third, fourth, and fifth abdominal sternites. The two vasa deferentia of the right testies run towards the left lateral side of the insect in an oblique latero-posterior direction, while the vasa deferentia of the left testis travel towards the right side, and on reaching the vicinity of the right testis turn dorsally and run parallel to the vasa deferentia of the right testis.

The common vas deferens in *Oryctes rhinoceros* continues as a thick muscular tube, which curves to enter the copulatory apparatus, the phallus. The ejaculatory duct varies from 7 to 10 mm. in length. At the time of copulation the ejaculatory duct becomes straight with the result that the phallus is pushed out.

(*b*) *External Genitalia*

Under the external genitalia are included besides phallus, the eighth, ninth and the tenth abdominal sternites with their derivatives. In pterygote males the accessory structures on the ninth abdominal sternite are present, while those of the tenth and eighth are absent.

In *Oryctes rhinoceros* the eighth sternite is reduced, but the invagination of its posterior part forms the genital chamber, which contains the phallus. In Coleoptera, generally the ninth sternite is either absent or present in the form of an ' X '-shaped structure, the spiculum gastrale. In *Oryctes rhinoceros* the ninth sternite is represented by the spiculum gastrale.

The phallus is an elongate, highly chitinised structure measuring about 15 mm. in length. It is divisible into two parts; a basal rounded phallobase (tegmen of Sharp and Muir, 1912), and a distal elongated cylindrical, ædeagus. The phallobase is highly chitinised and globular in shape, but the chitinisation does not extend to its ventral part, thus leaving a slit-like unsclerotised area. The ædeagus is also chitinised and consists of two arms at right angles to each other. The proximal arm is in continuation with the phallobase, while the distal arm is free. The distal area of the ædeagus is bifurcated. Posterior to this bifurcation, a soft muscular structure, the vesica, is protruded at the time of copulation. A small unsclerotised area on the dorsal part of the angles enables to strengthen out the distal arm of ædeagus. The bifurcated tips of the ædeagus act as titillators like the ' end plate ' of *Bruchus quadrimaculatus* (Mukerji and Bhuya, 1933).

IV. The Female Genitalia

(*a*) *Internal Genitalia*

In *Oryctes rhinoceros* there are two ovaries in the ventro-lateral position on either side in the abdomen. The ovaries of both the sides are similar in structure. Each ovary extends from the metathoracic segment to the pygidium and varies from 15 to 20 mm. in length. It is formed by the union of six ovarian tubules, which are arranged in two bundles; each bundle having three tubules in it. The bundles are held together by means of connective

tissue. The hold is further strengthened by numerous tracheæ which aerate the developing eggs.

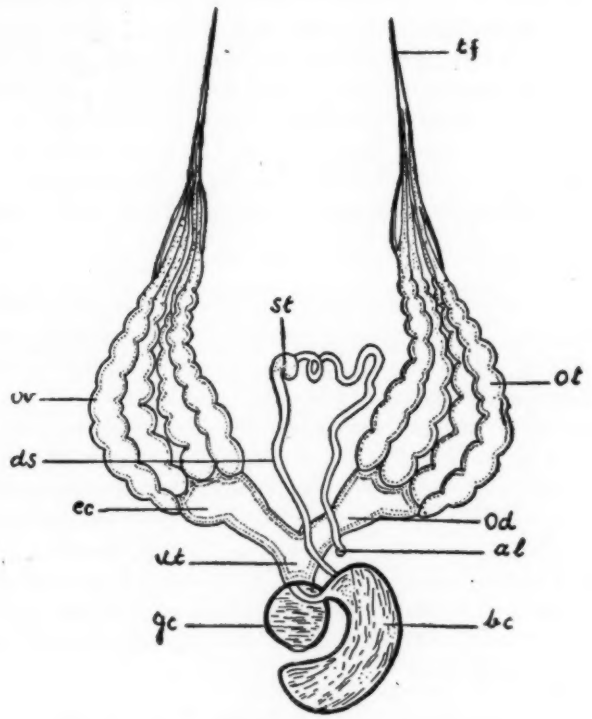
Each ovarian tubule consists of a number of eggs and groups of nutritive cells. The eggs are seen in the distal, while the nutritive cells in the proximal part, thus giving the ovary a perfect acrotropic condition. The eggs become smaller in size anteriorly and the tubules thus acquire a structure which is broad at its distal end but tapers anteriorly. Such forms of ovarian tubules likewise impart a similar shape to the ovary. So far as we have been able to ascertain, only Snodgrass (1935) and Imms (1957) have discussed the form of the ovarian tubules. Snodgrass (1935) assigns the polytropic form of ovarian tubule to Coleoptera in general, while Imms (1957) describes acrotropic condition in all polyphagous beetles. However, in *Oryctes rhinoceros* it is the acrotropic type of tubules which unite to form the ovary. Each ovarian tubule is divisible into two parts, the proximal terminal filament and the distal egg tube. The terminal filament is the anterior continuation of the tube and serves the suspensory function due to the presence of well marked ligament on its anterior position. The distal part is the egg tube which contains the germ cells and the nutritive cells. A distinct stalk, the pedicel (Snodgrass, 1935) is absent in *Oryctes rhinoceros*. All the ovarian tubules directly join with the expanded anterior end of the oviduct, the egg calyx.

The egg calyx is a small chamber of muscular walls situated on either side in the sixth abdominal segment. Generally, a single egg is found in the egg calyx, but the presence of two eggs is not a rarity. Mukerji and Bhuya (1937) in *Bruchrus quadrimaculatus* recorded the presence of two eggs in the lumen of the egg calyx. As soon as the eggs are discharged the calyx shows a remarkable reduction in its general form. The muscular walls relax to such an extent that sometimes it becomes difficult to distinguish between the calyx and the oviduct. The calyx narrows posteriorly to continue as the oviduct.

The oviduct of each ovary runs transversely in opposite direction between the sixth and seventh segments, so as to meet the centre to form a large common duct. The oviducts are small tubes ranging from 3 to 5 mm. in length. They are supplied with tracheal tubules. The common duct runs posteriorly in the middle; it takes up a curved course and thus appears like an arch. The formation of the common duct, its position and relation with other reproductive organs points to homologise it with the uterus of the higher animals. Metcalf (1933), in the female *Cytodrepa panicea* names a similar structure as the uterus. Mukerji and Bhuya (1937), Williams (1945) and Dutt (1958),

prefer to call it the common oviduct. However, in *Oryctes rhinoceros* the common duct can be named as uterus.

The bursa copulatrix in *Oryctes rhinoceros* is a thin-walled structure convex on its anterior side and concave on the posterior margin. The proximal end is narrow and bends towards the genital chamber. The bursa copulatrix extends from the sixth abdominal segment to the distal part of the pygidium and varies from 10 to 15 mm. in length.



TEXT-FIG. 2. The female genitalia of *Oryctes rhinoceros.*

In the lamiid beetle, *Nupserha bicolor*, Dutt (1958) describes a unique condition, where the common duct opens anteriorly into the bursa copulatrix. In *Oryctes rhinoceros* the bursa copulatrix has no direct connection like that one present in the lamiid beetle. Snodgrass (1935), Mukerji and Bhuya (1937) and Williams (1945) do not also agree with Dutt (1958) in regard to the connection of the uterus with the bursa copulatrix.

From the proximal region of the bursa copulatrix there arises a delicate tube, the ductus seminalis, which runs posteriorly. After traversing a little it runs transversely in the middle region forming coils between the two ovaries. At the formation of the first coil the tube dilates a little to appear like a chamber. The latter is termed as the spermatheca. The posterior end of the spermatheca narrows to continue as a tube which after taking two or three coils descends ventrally. The distal end of the tube dilates to form the accessory gland.

The uterus opens in a thin-walled globular structure, the genital chamber, situated in the eigth segment. At the proximal part of the genital chamber just anterior to the opening of the uterus in the former, the bursa copulatrix also opens in it through a delicate smooth-walled duct. The genital chamber is about 5 mm. in diameter in its middle; it reduces gradually at its posterior end to such an extent that a small slit, the gonopore, is left at the posterior extremity, and serves as the external opening of the chamber. The wall of the genital chamber is strengthened by a number of small sclerites.

(*b*) *External Genitalia*

The ovipositor and its associated structures form the external genitalia in female insects. In Coleoptera, the females are generally devoid of an ovipositor, Snodgrass (1935). In *Oryctes rhinoceros* outgrowths of the eighth and ninth abdominal segments form the reduced external genitalia. The ninth abdominal sternite in female Coleoptera generally bears the coxite and stylus as stated by Tanner (1927). In *Oryctes rhinoceros*, the reduced ninth segment is represented only by a small unsclerotised area surrounding the gonopore with no trace of its accessory appendages. The spiculum gastrale, an important derivative of the ninth segment, which affords a firm basis for muscular attachment, is also absent. The absence of external genital structures and spiculum gastrale seems to be correlated with the egg-laying habit of the female in a soft medium like dung and decaying organic matter. The tenth segment is absent.

V. Summary

1. Each testis is followed by two vasa deferentia, which join to form a common vas deferens.

2. The ejaculatory duct opens into the phallus.

3. The ædeagus is a curved organ, bifurcated at the distal end.

4. In the external genitalia of male, the eighth abdominal sternite is reduced; the ninth is represented by spiculum gastrale, whereas the tenth is

absent. In the female, the eighth is reduced, the ninth surrounds the genit a chamber, and the tenth is absent.

5. There are two ovaries, the egg tube is acrotropic.

6. The oviducts unite to form the uterus.

7. The spermatheca and the accessory gland join the bursa copulatrix which in turn opens into the genital chamber.

VI. Acknowledgements

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VIII. ABBREVIATIONS

ag, ædeagus; *al*, accessory gland; *bc*, bursa copulatrix; *cd*, common vas deferens; *ds*, ductus seminalis; *ec*, egg calyx; *ed*, ejaculatory duct; *ep*, epididymus; *gc*, genital chamber; *od*, oviduct; *ot*, ovarian tubule; *ov*, ovary; *pb*, phallobase; *st*, spermatheca; *sv*, seminal vesicle; *t*, tesis; *tf*, terminal filament; *ut*, uterus; *vd*, vas deferens.

THE USE OF SILICA GEL FOR THE ENUMERATION AND ISOLATION OF *AZOTOBACTER* IN SOIL

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While Winogradsky (1925 and 1926) used silica gel for the isolation of *Azotobacter*, Ashby (1907), Martin (1940) and Jensen (1940) used agar as the solidifying agent either for the plate count of *Azotobacter* or for the isolation of the same. In view of the cumbersome procedure involved in the preparation and sterilization of silica gel the use of agar was more popular. However, the agar that is usually available in bacteriological laboratories contains small amounts of nitrogen and as such even organisms, that do not fix nitrogen, may grow and form colonies. In attempts to enumerate *Azotobacter* from soil at I.A.R.I. different agar media Jensen (1940), Ashby (1907) and Martin (1940) were tried and it was observed that besides *Azotobacter*, colonies of several other organisms particularly of actinomycetes appeared on the agar plates.

Actinomycetes most frequently occurring on the agar plates was plated on plain agar (containing 0·3% N*) and it was found to be able to grow well on these plates. It did not fix any nitrogen in Jensen's liquid medium for *Azotobacter*.

Trials were therefore made to simplify the silica gel method so that it may be used for the enumeration of *Azotobacter* in the soil.

Method of preparation of silica gel.—Recently there has been a trend towards improving the method of preparation of silica gel. Taylor (1950) described a greatly improved method of the preparation of silica gel and Pramer (1957) has described a modified method. These methods require the use of an activated resin column and repeated adjustments of pH and concentrations, and are too cumbersome to be widely adopted. An attempt was made in this laboratory to simplify the procedure. Sterilisation by

* According to the specification laid by the Society of American Bacteriologists (*Manual of Microbiological Methods*,1957) the nitrogen in Bacteriological Grade Agar should not be more than 0·32%.

autoclaving was replaced by sterilisation with ultra-violet rays to avoid the formation of blow holes. The technique followed is as follows:—

A 20% solution of water-soluble crystalline sodium silicate is prepared by slow heating. This alkaline solution is cooled and filtered. A known volume of sodium silicate solution is then poured into a conical flask containing an equal volume of 33% HCl and the mixture thus prepared in small lots is then poured into sterilised Petri-dishes (12–15 c.c. for 9 cm. diameter dishes) and left undisturbed overnight for gelation to take place. The gel formed is transparent. These plates are then washed free from acid in running tap-water for about 48 hours. The pH of the draining water is tested by methyl red. The gel is freed from chlorides by keeping the plates immersed in distilled water which is changed after 5–6 hours till the washing is free from chlorides as tested by $AgNO_3$. This can be done usually in two to three washings. The pH of the gel is neutral at this stage. The plates are kept inverted on filter-paper sheets to allow the water to drain off. These plates are sterilized by exposure to ultra-violet light of 2437 Å wavelength at a distance of 70 cm. for ½ hour in the plating room.

For use of these plates in enumeration of *Azotobacter*, 2 c.c. of four times concentrated sterilised nutrient mixture (including $CaCO_3$) as contained in Jensen's agar medium are poured on the sterilised and chloride-free silica gel plates and water is allowed to evaporate by exposure of the plates to dust-free air in the plating room. Thereafter an exposure of an hour's duration to ultra-violet light in the closed plating room suffices to sterilise the plates. Scrapings from the plate were smeared on slides and stained with Rose Bengal and examined under the microscope. No organisms could be detected. Nutrient broth inoculated with these scrapings also failed to show any growth.

These plates are now ready to receive soil suspension inoculum of convenient dilutions. After pouring the soil suspension on the plates they are dried by exposure to dust-free air in a plating room sterilised with ultra-violet rays. Control plates (not inoculated with soil suspension) similarly exposed failed to show any growth in peptone broth or in Jensen's agar slants used for maintaining nitrogen fixing organisms.

The comparative development of *Azotobacter* and actinomycete colonies on Jensen's agar medium and silica gel medium (prepared as described here) from mixed cultures of these was ascertained in repeated experiments and it was seen that the silica gel media largely favoured the appearance of *Azotobacter* almost to the exclusion of others; development of other organisms was more in the agar medium in which *Azotobacter* was suppressed to a great extent (*see* Table I).

TABLE I

Comparative development of Azotobacter *and actinomycete colonies from mixed inocula on silica gel and Jensen's agar*

	Number of colonies (Average of four plates)	
	Azotobacter	Actinomycetes
Silica gel ..	11·5	None
Jensen's agar ..	0·5	30

The relative usefulness of the agar and silica gel plates for the enumeration of *Azotobacter* in soil was further tested by plating soil suspension from the same set of samples from a bacterization experiment with *Azotobacter* and phosphobacterin with wheat crop. As in other instances, no colonies, other than those of *Azotobacter*, appeared on the silica gel plates whereas the Jensen's agar plates showed colonies of other organisms as well as those of *Azotobacter*. The counts obtained are tabulated in Table II.

TABLE II

Count of Azotobacter *on silica gel and Jensen's agar medium from soil samples from pot culture experiment on wheat*

(Dilution 1 : 100)

Treatment	Silica gel *Azotobacter* (average of four plates)	Jensen's agar (Average of four plates)	
		Azotobacter	Other organisms
1. Control ..	82·25	11·25	190·25
2. Phosphobacterin ..	129·25	10·25	211·75
3. *Azotobacter* ..	125·75	6·5	292·75
4. *Azotobacter* + Phosphobacterin	92·00	10·5	224·00
5. F.Y.M. ..	87·00	8·0	150·25
6. F.Y.M. + Phosphobacterin	116·00	10·25	211·75
7. F.Y.M. + *Azotobacter* ..	107·50	4·25	235·50
8. F.Y.M. + Superphosphate + *Azotobacter* + Phosphobacterin	161·50	15·75	214·00

The counts obtained on agar plates were too low to be relied upon as the actinomycetes seriously hampered the correct enumeration of *Azotobacter*. The counts of *Azotobacter* obtained on silica gel plates were higher.

Pure cultures of *Azotobacter* were obtained by three successive platings on silica gel. The difficulty that is usually experienced in eliminating a closely associated rod form contaminant when plated on Jensen's agar or other medium has been easily overcome by employing silica gel.

SUMMARY

A simplified method for the preparation of silica gel is described. The gel has been found to be useful in enumerating *Azotobacter* population in the soil as only *Azotobacter* colonies appeared in the plates containing this gel. It is also possible to obtain pure cultures of *Azotobacter* by employing this technique.

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DISTRIBUTION OF PHOSPHATES IN THE SEDIMENTS OF THE KAKINADA BAY

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Introduction

The Kakinada Bay, on the East Coast of India, has been attracting the attention of oceanographers from various aspects. Hydrography in relation to plankton (Bhavanarayana, 1955); biology of mollusca (Narasimhasastry, 1955); the mechanical and chemical composition of sediments (Subba Rao, 1958); circulation pattern (Sivarama Sastry, 1958); the erosion of the beach at Uppada (La Fond and Prasada Rao, 1956); and the causes of the growth of sand spit north of Godavari confluence (Mahadevan and Prasada Rao, 1958) of this estuarine *cum* bay environment have been studied.

In an estuary or in any other marine environment, the productivity depends on the amount and distribution of nutrient salts such as silicates, phosphate, nitrates, etc., dissolved in the waters. It is accepted that the bottom sediments act as reservoirs of these nutrient salts. With this objective, the phosphate distribution in the bottom sediments of the Kakinada Bay in relation to mechanical composition, colour of the sediment and depth of the bay has been investigated and the results are presented in this paper.

Kakinada Bay

The Kakinada Bay, lying between Lat. 16° 51′ and 17° 03′ N. and Long. 82° 15′ and 82° 24′ E., is essentially a shallow estuary located at the mouths of the north-flowing distributaries of the river Godavari. The area under study is about 100 sq.miles. On the east side, the bay is cut off from the main sea by a long narrow sand pit which has grown off the delta in a north-south direction for about nine miles. The bay is connected on the north with the Bay of Bengal by a broad opening. On the western and southern sides the bay is bordered by deltaic alluvium and mud flats.

Sample collection.—Samples of bottom sediments from the lower part of the bay were collected during a cruise aboard I.N.S. Rohilkhand in 1956

with the aid of La Fond-Dietz Snapper. In 1957, and 1958 samples were collected from the upper part of the bay from a country boat by Mr. Subba Rao.

METHOD OF ANALYSIS

Phosphate occurs in sediments in the following forms, *viz.*, interstitial phosphate, adsorbed phosphate, organic phosphate and insoluble inorganic phosphate. The total phosphate is composed of all these forms of phosphates. The organic phosphate and the insoluble inorganic phosphate could not be estimated separately since the samples were not fresh. The other types of phosphates and the total phosphate are determined by the method of Denigès as modified by Robinson and Thompson (1948). Rochford (1951) has adopted this method with slight modifications to analyse the Australian estuarine sediments.

The method is based on the principle that when a sulphuric acid solution of ammonium molybdate is added to a soluble phosphate solution a complex phosphomolybdic acid is formed. Upon treatment with a reducing medium such as stannous chloride, the complex is reduced to a blue coloured substance believed to have the composition $H_3PO_4\ (4\ MoO_3 \cdot MoO_2)_2 \cdot 4\ H_2O$. The intensity of the colour developed is proportional to the concentration of the phosphate.

In order to bring the different types of phosphates in a soluble state, the first step is to prepare a 'silt-suspension' which in the present case is made to contain particles of size less than 0·062 mm. This has been achieved by dispersing a known weight of dry sediment in distilled water and wet-sieving this through 230 Endecotts mesh. The suspension was made up to one litre and used for analysis as detailed by Rochford (*loc. cit.*).

The contents of different forms of phosphate are recorded in Table I. The phosphate distribution is shown in Fig. 1.

OBSERVATIONS

An examination of the accompanying table of results warrants the following observations:

1. On the whole, the sediments of the Kakinada Bay have low contents of phosphate concentration. The total phosphate concentration varies from 10 μg.–50 μg. per gram of dry silt.

2. Barring the intertidal mud flats on the western side, the total phosphate concentration increases in the northern and western parts of the bay and decreases in the southern and eastern parts.

TABLE I

Distribution of phosphates in the sediments of the Kakinada Bay

(1) Station Numbers	(2) Year of collection	(3) Mechanical composition of the sediment	(4) Colour of the sediment	(5) Interstitial phosphate (μg./g. of dry sediment)	(6) Adsorbed phosphate (μg./g. of dry silt)	(7) Total phosphate (μg./g. of dry silt)
525	1956	Silty clay	Green	1	7	19
529	,,	,,	Greyish green	2	3	30
554	,,	Sand	Light green	1	3	31
557	,,	Silt clay	Grey	1	5	39
559	,,	,,	Greenish grey	2	5	21
562	,,	Sand-silt-clay	Grey	1	3	31
563	,,	Clay	Greenish grey	1	11	27
564	,,	Silty clay	Greyish green	2	11	20
569	,,	,,	Green	1	2	21
1	1957	Sandy clay	Ash grey	0	2	18
5	,,	Clayey sand	Grey	1	5	19
11	,,	Silty clay	Green	2	2	10
13	,,	,,	Dark grey	2	4	17
17	,,	Sand-silt-clay	,,	3	4	19
19	,,	Clayey sand	,,	2	5	15
22	,,	Silty clay	Grey	1	12	35
26	,,	,,	Dark grey	3	11	21
28	,,	,,	Dark grey	1	4	23
30	,,	,,	Brownish grey	4	5	16

TABLE I (*Contd.*)

(1) Station numbers	(2) Year of collection	(3) Mechanical composition of the sediment	(4) Colour of the sediment	5) Interstitial phosphate (μg./g. of dry sediment)	(6) Adsorbed phosphate (μg./g. of dry silt)	(7) Total phosphate (μg./g. of dry silt)
35	„	Sandy clay	Brown	0	13	45
42	„	Silty clay	Dark grey	0	7	26
46	„	„	Greenish grey	1	5	15
50	„	Sandy clay	„	2	11	15
54	„	„	Dark grey	0	5	12
58	„	Silty clay	Brownish grey	0	10	32
61	„	Sand-silt-clay	Grey	2	15	40
65	„	Sand	Brownish grey	1	10	26
69	„	Silty clay	Brownish green	1	3	25
71	„	Clay	„	3	4	21
72	„	Silty clay	„	0	5	25
74	„	„	„	0	4	23
75	„	Sand-silt-clay	„	2	6	28
80	„	Clay	„	0	15	30
85	„	Silty clay	Grey	1	12	50
91	„	Sandy clay	Brownish grey	1	10	40
96	„	Clayey sand	Greenish grey	2	10	27
99	„	Silty clay	„	1	12	45
105	„	Sand-silt-clay	Dark grey	2	9	40

TABLE I (*Contd.*)

Station numbers	Year of collection	Mechanical composition of the sediment	Colour of the sediment	Interstitial phosphate (μg./g. of dry sediment)	Adsorbed phosphate (μg./g. of dry silt)	Total phosphate (μg./g. of dry silt)
113	,,	Silty clay	Greyish green	6	15	43
121	,,	Clayey sand	Grey	3	12	20
124	,,	Silty clay	Brown	2	20	25
129	,,	,,	Greyish green	2	5	25
131	,,	Sand-silt-clay	Brown	0	3	10
133	,,	Sand-silt-clay	Brownish grey	0	2	11
137	,,	,,	Greyish green	2	3	16
130	1958	Silty clay	,,	1	11	46
132	,,	,,	Greyish brown	1	5	40
133	,,	Clay	Greyish green	1	4	37
134	,,	Sand-silt-clay	,,	1	9	45
142	,,	Silty clay	,,	0	3	21

3. There is variation, though in small degree, in the amounts of different types of phosphates from station to station.

4. The mechanical composition and the colour of the sediment apparently bear no relation to the phosphate accumulation in the sediments.

5. The depth of the bay seems to have no controlling influence on the phosphate concentration in the bottom sediments.

DISCUSSION

High values of phosphates have been reported in the sediments of Australian estuaries (Rochford, 1951, 1958) and in the marine basins of Southern California (Rittenberg *et al.*, 1955). Seshappa (1953) and Seshappa

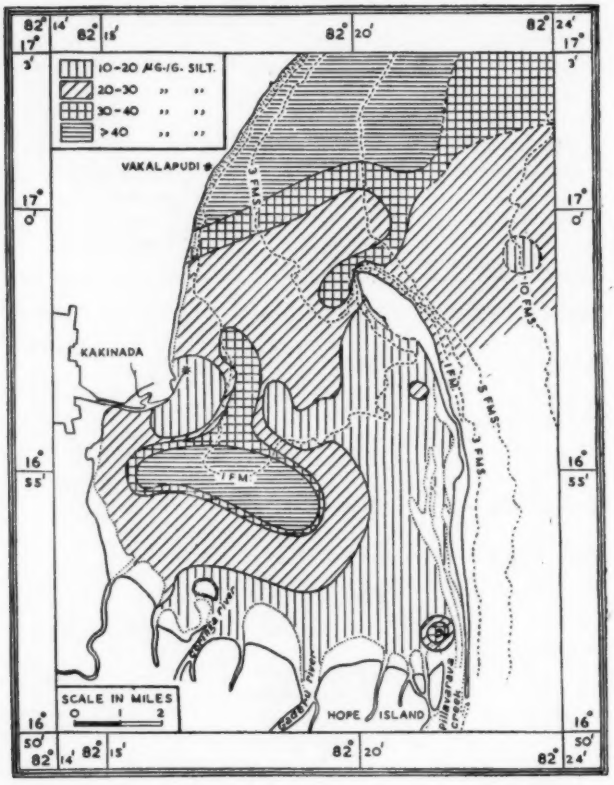

FIG. 1. Distribution of Phosphate in the Sediments of the Kakinada Bay.

and Jayaraman (1956) also obtained very high values of interstitial phosphate, adsorbed phosphate and total phosphate in the muds of Malabar Coast. Suryanarayana Rao (1957) recorded very high values of total phosphate and inorganic phosphate in the inshore waters off West Hill on the Malabar Coast. These observations suggest that waters with high phosphate content are underlaid by sediments of high phosphate content. A similar correspondence has been found in the present investigation where the sediments of low concentration of phosphates closely agree with overlying waters of poor phosphate content (*i.e.*, 0·33 μg. in September to 2·6 μg. in March, of phosphate per litre of the waters; Bhavanarayana, *loc. cit.*). The low content of phosphates in these sediments and the overlying waters as well may be traced to the insignificant contributions of the Godavari waters. The

investigations of the Godavari waters, presently being conducted by the Zoology Department, Andhra University, corroborates this view. Maximum dilution of the bay waters has resulted in the dilution of phosphates.

In the northern parts of the bay the enrichment of phosphates can be attributed to the mixing of silt-laden waters of the rivers with the saline waters of the sea. On the seaward side of the spit the waves break very heavily and huge quantities of sediments are brought into suspension and carried away by the littoral currents. As soon as it is brought into the deep waters north of the spit, the material is deposited. The silt-laden river-waters are believed to be the source of phosphates. Barring the intertidal mud banks on the western side the rest of the bay is also enriched in phosphates. This region is characterised by the pungent smell of H_2S (Narasimhasastry, *loc. cit.*) which is indicative of large-scale mortality of organisms. This type of large-scale mortality, at the commencement of monsoons, is advocated as one of the possible ways of enrichment of phosphate in overlying waters (Seshappa and Jayaraman, *loc. cit.*). The tidal waters which wash the banks might, if aided by local winds, churn up the bottom sediments and bring into suspension the phosphates which when brought into deeper and calmer waters during the ebb get precipitated. Narasimhasastry (*loc. cit.*) and Subba Rao (*loc. cit.*) have observed that fauna is mostly confined to the southern and western parts of the bay. It is an index of the abundant availability of nutrients including the phosphates. This results in the impoverishment of phosphates due to consumption by the populations in these parts. On the southern and eastern sides of the bay the impoverishment of the phosphate owes much to the greater velocity of the currents at the rivermouths which carry away the particles of the sediments and along with them the phosphates.

The variation in the values of the interstitial phosphate, adsorbed phosphate and total phosphate contents from station to station may be due to several factors. The adsorbing power of the mud is a major factor, which in turn is determined by the content of silt-colloidal materials and the presence of organic complexes containing iron (Ohle, quoted by Rochford, 1951). Again, both interstital phosphate and adsorbed phosphate are utilized by the mud biota and the phosphate uptake of the mud biota is determined by the pH, redox potentials, oxygen content and other properties of the mud itself (Zobell, 1946). It has been noticed in the Kakinada Bay that the seasonal high and low in the phosphate content in waters is coincident with the decay and the bloom of the plankton respectively (Bhavanarayana, *loc. cit.*).

Klenova and Budianskaya (1940) found that in some regions of Kara Sea the quantity of phosphorus increased with the increase in the particle

size of the grain while in other places a greater fineness of materials was associated with the increase in phosphorus content. They further observed that no direct relation could be established between the phosphorus accumulation and the colour of the sediment. In the case of the Kakinada Bay too, no direct or indirect relationship has been observed between the mechanical composition and the colour of the sediment, and the phosphate accumulation in the sediments.

The shallow nature of the bay does not seem to have any effective controlling influence on the accumulation of phosphates whereas Klenova and Budianskaya (*loc. cit.*) have reported an increase of phosphorus in the regions of shallow waters.

Rochford (1951, 1958), Seshappa (1953), Rittenberg *et al.* (1955) and many others came to the conclusion that part of the nutrient phosphates in the water masses is contributed by the underlying sediments. Cellular circulation and recirculation due to the dilution of estuarine waters by freshwaters (Rochford, 1951), increased agitation of waters down to the bottom due to strong winds during monsoons (Seshappa, *ibid.*) and upwelling of waters are possible mechanisms which can bring about this type of exchange of nutrients between the sediments and the overlying waters. But the winds, even during monsoons, are not very strong here. About 75 miles north of Kakinada, off Visakhapatnam Coast, it has been found that upwelling of waters replenishes nutrient phosphates to the surface (Bhavanarayana and La Fond, 1957). But Murty (1955) found that no such upwelling of waters occurs in the Kakinada Bay. It is concluded, therefore, that the contribution of phosphates to waters from sediments below, which are inherently poor in phosphates, cannot be appreciable.

SUMMARY AND CONCLUSIONS

Fifty bottom sediment samples of Kakinada Bay have been analysed for their phosphate content with the object of studying the distribution of the different types of phosphates in relation to the mechanical composition, the colour of the sediment and the depth of the bay. It has been found that, the phosphate content of bottom sediments is very low; there is in general an increase in the concentration of total phosphate in northern and western parts of the bay and a decrease of the same in the southern and the eastern parts; the intertidal mud flats are also poorer in phosphates; there is no apparent relationship existing between the mechanical composition and phosphate concentration, or the colour of the sediment and the phosphate concentration and the shallow nature of the bay seems to have no effective

controlling influence on the phosphate accumulation. The replenishment of phosphates in the waters from the sediments below does not seem to be appreciable in the case of the Kakinada Bay.

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DIFFERENTIAL FLUORESCENCE OF THE CHROMOCENTERS AND NUCLEOLAR EQUIVALENTS OF THE YEAST NUCLEUS IN ACRIDINE ORANGE

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Introduction

The living nucleus of yeast shows formed structures within the area enclosed by the nuclear membrane (Henneguy, 1896; Royan, 1956 *a*, *b*, 1958 *b*, *c*; Thyagarajan and Subramaniam, 1957 *a*, *b*; Thyagarajan, 1959; Aswathanarayana and Subramaniam, 1958; Subramaniam, Royan, Thyagarajan, Aswathanarayana and Subramanyam, 1959). These organelles could be stained with hæmatoxylin (Royan, 1956 *a*, 1958 *a*; Thyagarajan and Subramaniam, 1957 *b*; Aswathanarayana and Subramaniam, 1958). Some of them were Feulgen positive. The Feulgen negative structures were suspected to be the nucleolar equivalents. Cells fixed in iodine-formaldehyde-acetic acid solution and stained with Giemsa, without any hydrolysis, showed a differential staining of these two organelles (Royan, 1958 *c*). The chromocenters were red while the nucleolar equivalents were blue.

These conclusions drawn from fixed preparations necessitated confirmation, if possible, by vital staining. If examination of material was to be limited to observations with an ordinary microscope, then, no vital stain capable of such a distinction is available. The fluorochrome, acridine orange, has been claimed (Armstrong, 1956; Smiles and Taylor, 1957; Bishop and Austin, 1957; Bishop and Smiles, 1957 ; Austin and Bishop, 1959) to enable a distinction of the cell organelles by their differing fluorescence. An attempt was, therefore, made to elucidate whether the intranuclear structures of yeast exhibit a differential fluorescence when stained with acridine orange.

Material and Methods

The earlier observations on the strain, *Saccharomyces cerevisiæ*, NRRL, Y. 567 (Royan and Subramaniam, 1956 ; Royan, 1956 *a*, *b*, *c*, 1958 *a*, *b*, *c*) were carried out using barley malt wort of sp. gr. 1·020 and pH 4·6–4·8. During an exploration of alternative media suitable for observations on

the nucleus in the living condition, it was discovered that a medium (MPYA) having the composition, maltose (City Chem. Corp., N.Y.) 10 gm., peptone (Difco) 1 gm., yeast extract (Difco) 0·5 gm. and asparagine (Nutritional Biochem. Corp., N.Y.) 0·2 gm. dissolved in 100 ml. of distilled water and the specific gravity of which was adjusted to 1·020 and the pH to 4·6–4·8 would serve equally well. Unlike in malt wort, the nuclei were visible only in a smaller percentage of cells from the eleventh day onwards. Photos 1 and 2 show cells with nuclei from a 6-day barley malt wort culture and from a 13-day MPYA medium respectively. There is no major difference in the structural details of the nuclei.

Though the ideal temperature for culturing the strain is 25° C. the progress of aging, after the nuclei have become visible, was rapid with a consequent granulation of the cytoplasm. This aging process was slowed down by transferring the 11–12 day old cultures to a refrigerator (5–7° C.).

The source of ultra-violet light was a Zeiss super pressure mercury lamp with a heat filter, a blue energizing filter and an orange-coloured check filter for the eye-piece. The blue filter is said to permit essentially the 4 g lines at 366, 403, 405 and 436 mμ as fluorescent energizing light to pass through. The microscope used for examination had a condenser 1·4 N.A and an objective × 97 with an iris diaphragm. The stained preparations were photographed with a Leitz Mikro attachment giving a 1 : 1 magnification on Kodak Microfile and Ektachrome films.

OBSERVATIONS

Acridine orange (E. Gurr) was dissolved in pyrex distilled water. The determination of the optimal concentration of the dye was by testing a series of dilutions. When the concentration of the dye was 1 in 20,000, the staining was rapid and the cytoplasm assumed quickly a reddish orange fluorescence. It was only in dilutions above 1 in 30,000 that the staining was relatively slow. A dilution of 1 in 40,000 was preferred because the chromocenter was green while the nucleolar equivalent was orange. A concentration suitable for cells grown in MPYA medium was found to stain some of the cytoplasmic granules reddish orange in those grown in barley malt wort.

The procedure adopted was to mount a few cells in a drop of acridine orange (1 : 40,000) and flatten them under the coverslip thirty minutes later, by removing the extra stain with filter-paper strips. They were then sealed with paraffin wax, kept in the dark and examined periodically.

Another method was to centrifuge out the yeast cells from 1 ml. of a well-shaken 10–14 day MPYA culture and suspend them in 5 ml. of acridine orange (1 : 40,000) in a test-tube. After a stay of 24 hours in the dark, samples from the stain were sealed under a coverslip with paraffin wax and examined periodically. When mounted, the cells, with the exception of the dead and senescent ones, were green and exhibited no orange fluorescence. The nuclear details were clear owing to their differing intensities, the nucleolar equivalent being prominent. It was only after the lapse of 24 hours after mounting under the coverslip that a differential fluorescence of the nucleolar equivalents and chromocenters was observed.

Transfer of cells stained for 24 hours in a test-tube, to fresh growth medium without the stain, resulted in cell proliferation. The cell deposit at the bottom of the tube 24 hours after transfer, was light orange in colour. Examination revealed the persistence of the dead and senescent cells fluorescing orange. The budding cells were dull green.

The first procedure was preferred owing to the shorter time required for the differential fluorescence to appear in the cells. It should be emphasized that not all cells show a uniformity in the nature of the polychromatic fluorescence. If the cells were very few, they showed an orange halo around them on mounting which disappeared subsequently. The cytoplasm of the majority of such cells was green at the beginning. Many of them turned yellow and quickly assumed a bright orange fluorescence. Even then the chromocenters remained green.

If there were a large number of cells under the coverslip, the majority remained green with clear cellular details. Only a few showed areas fluorescing orange. The optimum number of cells to be mounted under a coverslip could be determined only by experience. Curiously enough, the nucleus visible under phase-contrast only in a small percentage of cells stained in acridine orange (1 : 40,000) was clear in the majority of the cells under ultraviolet illumination. The progress of staining under optimal conditions was followed repeatedly.

The following sequence of events were observed in material mounted in acridine orange (1 : 40,000). A few minutes after sealing, some cells orange in colour put in their appearance. These seem to be the senescent or dead cells. The others could at first be located only as green patches with clear outlines. There was a gradual increase of the green fluorescence of the cytoplasm. The nucleolar equivalent put in its appearance as a greenish white structure. It was at this stage that the vacuole became visible as an

unstained area on a dark background. The nuclear membrane then became distinct. The chromocenters were the last to appear as structures fluorescing green. The nucleolar equivalent began slowly to show an orange fluorescence whose intensity increased with time. In some cells, however, the cytoplasm was light orange, the nucleolar equivalent bright orange, the chromocenters alone remaining green. The nuclear membrane was of the same colour as the cytoplasm. The time required for the above series of changes resulting in a clear polychromatic fluorescence of the nuclear details ranged from three to twenty-four hours.

If the cells were not exposed to ultra-violet irradiation, this characteristic polychromatic fluorescence could be observed even after the lapse of five days, if the slides were kept shielded from light. Very short exposures, as happens when a slide is scanned quickly, does not produce any loss in intensity of the fluorescence of the structures. But if specific regions are exposed for periods of more than one minute there was a tendency for the loss of the orange colour in some of the cells. These became green or greenish yellow.

The loss in intensity on exposure to ultra-violet light created difficulties for photography. The Microfile film used being a slow one, the exposure times ranged between two and a half to four minutes. Even though cells fluorescing brightly were chosen, there was a loss in intensity of the orange tint on the completion of the exposure. The pictures were better on the faster Ektachrome film. Photos 3 and 4 printed from Microfile negatives illustrate the condition on staining with acridine orange. The vacuole (V) could be seen as an unstained area in Photos 3 and 4. The nucleolar equivalant (NE) appears as a characteristic crescent attached to the nuclear membrane (NM) in the illustrations (compare with Photos 1 and 2). The chromocenter (C) lies free in the nuclear matrix.

DISCUSSION

Yasaki and Sinoto (1952) could not observe the nucleus of *Saccharomyces cerevisiæ* when stained with the fluorochrome, aluminium morine. Krieg (1954) claimed that he could stain the organelle, identified by Rochlina as the nucleus of the yeast cell, uniformly with acridine orange. Rustad (1958) saw the nucleus of *Schizosaccharomyces pombe* as a vacuole of low refractive index in living cells and reported that it gave an intense green, yellow-green or yellow fluorescence. He did not observe any differential fluorescence in the nucleus.

The earlier distinction of the nucleolar equivalents and chromocenters in the yeast nucleus was by their differing affinity for the components of the

Giemsa stain. The chromocenters were red while the nucleolar equivalents were blue (Royan, 1958 *c* ; Subramaniam *et al.*, 1959 ; Subramaniam, 1960). These two organelles could equally well be distinguished using acridine orange. The chromocenters fluoresced green while the nucleolar equivalents were orange.

Summary

The nucleus with its limiting membrane and organelles was visible in the majority of the yeast cells stained vitally with the fluorochrome, acridine orange, at a dilution of 1 in 40,000. The intra-nuclear structures could be distinguished by their differential fluorescence. The chromocenters were green while the nucleolar equivalents were orange. The vacuole showed no fluorescence.

Acknowledgments

One of us (S. R.) is grateful to the University Grants Commission for the award of a Senior Research Fellowship. The technical assistance of Mr. N. S. Kumar in testing the suitability of the MPYA medium for a study of the living nucleus is acknowledged.

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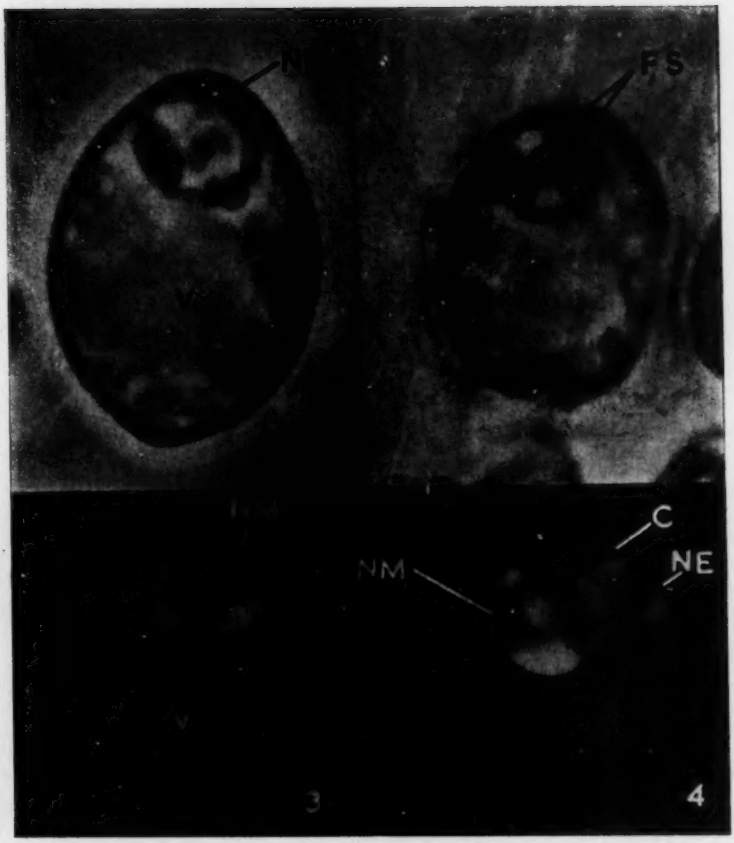
EXPLANATION OF PLATE IX

PHOTO 1. Living Cell (Barley Malt Wort), Ordinary illumination. × *ca.* 6,200.

PHOTO 2. Living Cell (MPYA Medium), Ordinary illumination. × *ca.* 6,200.

PHOTOS 3 and 4. Cells treated with a solution of acridine orange (1 : 40,000), × *ca.* 5,400. Note the nucleus with the distinct nuclear membrane (NM) enclosing the chromocenter (C) and the nucleolar equivalent (NE).

C, Chromocenter; FS, Intra-nuclear Structures; NE, Nucleolar Equivalent; NM, Nuclear Membrane; V, Vacuole.



PHOTOS 1–4

SALINITY TOLERANCE AND OXYGEN CONSUMPTION OF THE CATTLE LEECH *HIRUDINARIA GRANULOSA*

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(Communicated by Dr. H. Srinivasa Rao, F.A.SC.)

While there is considerable information on the effects of dilute media on marine and brackish water animals, there is little information on the influence of media of increasing osmotic concentration on the activity and metabolism of freshwater animals (Pampapathi-Rao, 1959). It has been demonstrated by several workers (*vide* Prosser, 1950; Pampapathi-Rao, 1958 *a*; Lockwood, 1959) that many freshwater forms will survive for various lengths of time in dilutions of sea-water. Most of these studies are concerned with the freshwater crustaceans and fishes. Other freshwater organisms such as some of the leeches have received no attention in this regard. While studying the influence of salinity on the oxygen consumption of some tropical poikilotherms, it was noticed that the cattle leech, *Hirudinaria granulosa*, could withstand direct transfer to sea-water. This observation prompted the study of the response of this form to the media of increasing salinity, the results of which are reported below.

Material and Methods

Hirudinaria granulosa is a common Indian cattle leech found in freshwater ponds and sluggish streams feeding on the blood of cattle and other vertebrates. These occur in large numbers in most freshwater ponds around Madras. The leeches were collected and kept in the laboratory in tap-water at room temperature which varied between 29° C. and 31° C. They were acclimatized to sea-water in batches of three at a time starting with 10% (3·24‰) sea-water and gradually going up the following media prepared by dilution of sea-water (32·4‰) with tap-water:

20% (6·48‰);	30% (9·72‰);	40% (12·96‰);
50% (16·20‰);	60% (19·44‰);	70% (22·68‰).

In each grade of salinity the leeches were allowed to remain for two days (48 hours) before being transferred to the next higher medium. Bottles of the same size were taken and they were fully covered with black paper to avoid the influence of light. The temperature during the course of these experiments varied between 29° C. and 31° C. None of the leeches were fed after arriving in the laboratory.

RESULTS

Till the leeches were transferred to 50% sea-water no apparent ill-effects were noticed. In 60% sea-water the movements became slow and they were found to be inactive. It was also found that the leeches could survive only for 2–4 days in this medium. Even in 50% sea-water they lived only for 4–5 days. On transfer to 70% sea-water all leeches died within 10–15 hours.

Another batch of leeches was transferred to different salinities (10%, 20%, 30%, 40% and 50% sea-water) directly from tap-water. It was observed that the leeches in 50% sea-water died within 10–20 hours and those in 40% sea-water died in 2–4 days. The leeches in 10%, 20% and 30% sea-water could live indefinitely. This shows that the maximum survival concentration was between 30% and 40% sea-water.

To note the influence of salinity on the gross weight of the animal, 8 leeches of different sizes were weighed after allowing them to remain for 30 minutes in the following media of salinity: 10%, 20%, 30%, 40%, 50%, 60% and 70% sea-water. The transfer was gradual from a lower to the next higher salinity. It was noticed that there was a gradual loss of weight in all cases (Table I and Fig. 1). Before weighing, the leeches were blotted to remove surface water and the weights were taken with a sensitive balance to the nearest milligram.

The loss of weight was also determined by keeping leeches in the different media of salinity after direct transfer from tap-water. In media of higher salinity (60%, 50% and 40% sea-water) the loss of weight was rapid till they died. In other media it was found that the leeches after an initial rapid loss of weight gradually reached a steady level and later this weight has been almost maintained (Table II and Fig. 2).

After a sojourn in dilutions of sea-water for about 2 days the leeches were directly transferred to tap-water. The weights were taken periodically and it was seen that they regain some weight though they do not recover the original weight in freshwater. It is seen that this recovery is greater after treatment with higher salinities.

TABLE I

Hirudinaria granulosa—*Weights of eight individuals after allowing them to remain for* 30 *minutes in each medium. The change was gradual from one to the next medium of higher salinity*

Sl. No.	Weight in grams after 30 minutes in							
	T.W.	10%	20%	30%	40%	50%	60%	70% S.W.
1	1·44	1·35	1·27	1·07	0·87	0·75	0·71	0·69
2	1·59	1·51	1·41	1·24	0·99	0·85	0·78	0·75
3	1·70	1·49	1·44	1·26	1·04	0·93	0·87	0·83
4	1·89	1·80	1·73	1·19	1·15	1·03	0·94	0·89
5	2·33	2·17	2·09	1·85	1·49	1·29	1·16	1·11
6	5·75	5·63	5·41	5·13	4·50	3·85	3·31	3·03
7	6·03	5·80	5·76	5·33	4·70	3·97	3·63	3·48
8	9·78	9·69	9·63	9·08	8·03	6·93	6·14	5·65

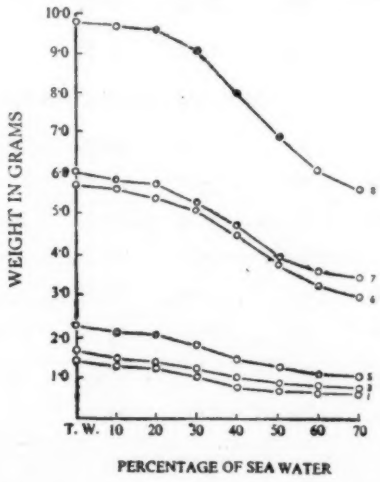

FIG. 1. Weight of the individual as a function of the salinity of the medium, in six specimens of *Hirudinaria granulosa*. Numbers on the right-hand side refer to the serial number of the individual listed in Table I.

TABLE II

Weights of Hirudinaria granulosa *taken after direct transfer from tap-water to different media of salinity. After* 48 *hours' stay in salt-water, they were transferred to tap-water and the weights recorded at intervals*

Date	Time (hrs.)	Number of hours	Weight in grams in				
			T.W.	10%	20%	30%	40%
10–12–1959	10–00	0	3·24	12·45	11·12	2·23	5·42
	11–30	1½	3·12	11·61	10·70	2·05	4·13
	13–00	3	3·11	11·61	10·40	1·71	3·81
	14–30	4½	3·10	10·96	10·17	1·69	3·61
	16–00	6	2·95	10·25	9·99	1·57	3·52
11–12–1959	10–30	24½	3·03	10·12	9·89	1·70	3·54
	12–00	26	3·21	9·97	9·79	1·63	3·49
	13–30	27½	3·22	9·70	9·60	1·62	3·53
	15–00	29	3·23	9·82	9·51	1·59	3·60
	16–30	30½	3·12	9·83	9·50	1·68	3·66
12–12–1959	10–00	48	3·21	9·84	9·51	1·74	3·60
			After reversal direct to tap-water				
	11–00	49	3·20	10·29	10·39	1·86	4·45
	12–00	50	2·94	10·30	10·47	1·91	4·65
	13–00	51	2·93	10·35	10·37	1·97	4·75
	15–00	53	2·87	10·20	10·38	1·99	4·81
	16–00	54	2·90	10·32	10·39	2·01	4·75
	17–00	55	2·95	10·41	10·40	1·99	4·76
13–12–1959	10–00	72	3·10	10·40	10·42	2·01	4·81
	18–00	80	3·05	10·35	10·30	2·01	4·82

In six individuals influence of salinity of the medium on oxygen consumption was studied. The oxygen consumption in tap-water, 25%, 50%, 75% and 100% sea-water was measured using the method described by Saroja

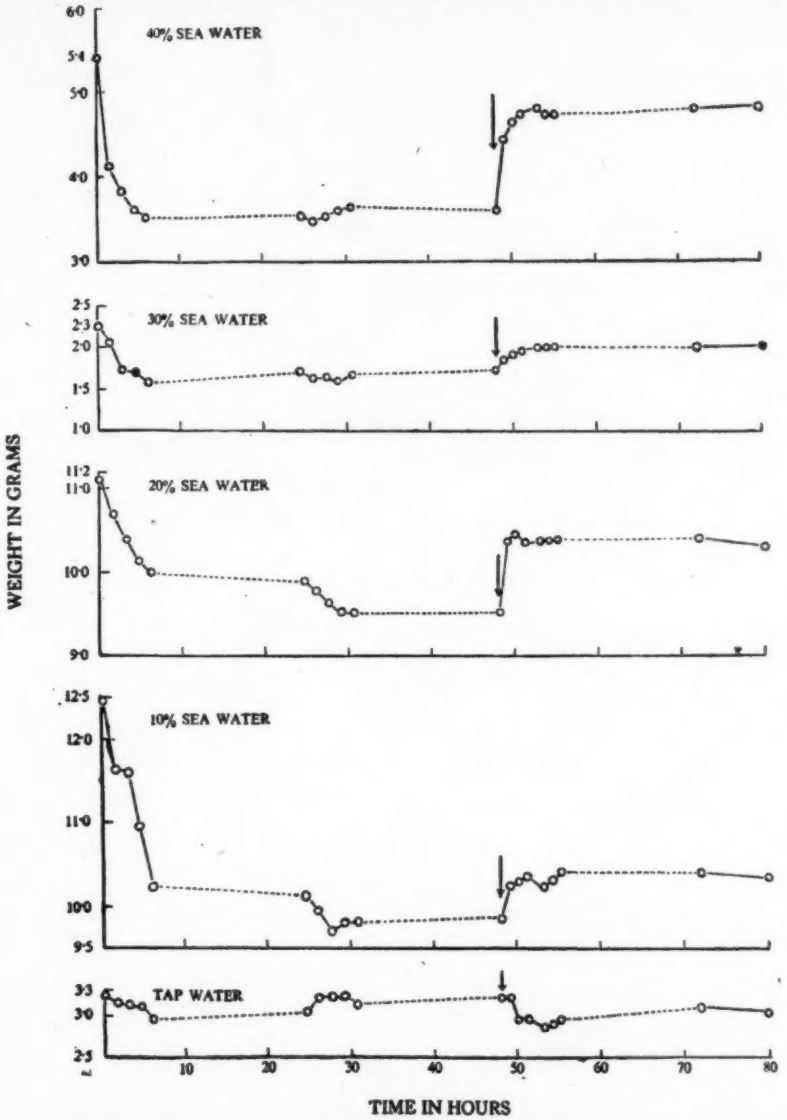

FIG. 2. Changes in the body weight of individual *Hirudinaria granulosa* as a function of time in media of different salinities. Arrow indicates the point when the leeches were transferred back to freshwater from the experimental media.

(1959). In each respiratory chamber two leeches were kept and their total oxygen consumption and the weight were noted (*vide* Table III and Fig. 3).

TABLE III

Oxygen consumption of Hirudinaria granulosa *in different salinities*

Expt. No.	No. of leeches in resp. chamber	Total weight gm.	Oxygen consumed (ml./hr.) in				
			T.W.	25%	50%	75%	100% S.W.
1	2	14·09	0·732	1·024	0·774	0·292	0·293
2	2	9·76	0·732	1·170	0·774	0·438	0·146
3	2	9·49	0·585	0·732	0·584	0·585	0·146

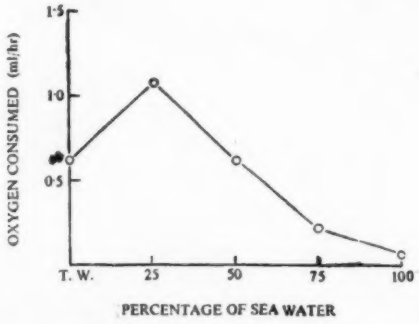

FIG. 3. Oxygen consumption as a function of the salinity of the medium in *Hirudinaria granulosa* (values taken from Table III).

In each new medium, leeches were allowed to remain for 15 minutes before readings were started. It was observed that the rate of oxygen consumption showed an increase in 25% sea-water and a gradual decrease with further increase of the salinity of the medium. The maximum oxygen consumption was noticed in 25% sea-water while the minimum was in 100% sea-water.

REMARKS

It has been shown above that the common cattle leech which occurs in freshwater is capable of surviving in a salinity of 12·96‰, if acclimatized gradually to increasing salinities. The fact that there is a rapid loss of weight in salinities above this appears to show the failure of the osmoregulatory mechanism in these media, resulting, perhaps in increased concentration of body fluids due to loss of water, and this leads to death. But in media less

concentrated than 12·96‰ there is an initial rapid loss of weight with subsequent stabilization of weight indicating an initial loss of water, perhaps till isotonicity with the medium is reached. But it is seen that little weight is regained by transfer to freshwater after prolonged stay in 10% or 20% sea-water, while considerable weight is regained after similar treatment in 30% or 40% sea-water. This regain of weight, only after a stay in the higher salinities indicates that inward permeability to water is slower and requires steeper osmotic gradients between the inside and outside as compared to the outward permeability to water. This decreased inward permeability to water in a freshwater organism is of definite adaptive value osmotically.

The pattern of oxygen consumption in different media is of interest. It is seen that the highest oxygen consumption is in 25% sea-water. It has been shown above that even 10% sea-water is slightly hypertonic to the leech. The increased oxygen consumption in 25% sea-water may therefore be a result of the osmotic stress imposed by the hypertonicity of the medium. Similar observations have been reported in certain crustaceans by Lofts (1956) and Pampapathi-Rao (1958 *b* and 1959). But the fact that the oxygen consumption falls steeply in media of higher salinities, such as 50%, 75% and 100% sea-water might be explicable in terms of the lethal effects of the increased concentration of the body fluids due to a rapid loss of water from the animal as shown above.

Summary

1. It has been shown that the common Indian cattle leech, *Hirudinaria granulosa* is capable of surviving indefinitely in a salinity of 12·96‰ if acclimatized gradually to increasing media of salinity.

2. The maximum survival concentration lies between 30% and 40% sea-water (salinity 32·4‰).

3. *Hirudinaria granulosa* loses weight when transferred to dilutions of sea-water. In media of lower salinity it was found that after an initial rapid loss of weight the leeches gradually reached a steady level and this weight was maintained. Return to freshwater did not result in complete recovery of the original weight.

4. The influence of salinity on oxygen consumption was studied. It was noted that the maximum oxygen consumption was in 25% sea-water while the minimum was in 100% sea-water.

Acknowledgements

My grateful thanks are due to Dr. Kandula Pampapathi-Rao, Head of the Department of Zoology, Sri Venkateswara University, Tirupati, for

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SOME OBSERVATIONS ON THE RATES OF PROTEOLYSIS DURING FLUE-CURING OF TOBACCO LEAVES*

By A. S. Sastry ‡

Received October 12, 1959

(Communicated by Dr. B. Viswa Nath, F.A.SC.)

During recent years some investigations have been conducted on the progressive chemical changes during flue-curing.[1-4] However the rates at which the various reactions take place and the causes for the same are largely not known.

The only work, known to the author, where an attempt has been made to study the rates of proteolysis during flue-curing is that of Askew *et al.* (*loc. cit.*). They examined some of the data of the author[2] quoted by Bacon *et al.*[4] and came to the conclusion that the increase in reducing sugars during flue-curing was directly proportional to the amount of protein nitrogen hydrolysed. The entire data of the author were however not available to them. Their own data also appeared to conform to such a relationship.

The purpose of the present note is to examine the entire data of the author[2] and of Askew[1] in the light of the latter's observations and the present-day concepts of protein metabolism in the plant. An interesting relationship between protein and soluble nitrogen, observed by the author in the various sets of leaves, will also be presented as it promises to offer a plausible explanation for the differences in the rates of proteolysis in these leaves.

Material and Methods

The material and methods are the same as those described by the author[2] previously. Four curings of Harrison Special variety of flue-cured tobacco grown on the unmanured plots of the Tobacco Research Station, Guntur, have been studied. They are as follows:—

1. Curing A .. 2nd primings, 1944.
2. Curing B .. 3rd primings, 1944.
3. Curing C .. 6th primings, 1945.
4. Curing D .. 7th primings, 1945.

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* This paper was read at the First Conference of Tobacco Research Workers held at Bangalore on 31-1-1957 and 1-2-1957.

‡ Biochemist, Central Tobacco Research Institute, Rajahmundry, India.

RESULTS AND DISCUSSION

The changes in protein nitrogen with time in the four curings under reference 2 are shown graphically in Fig. 1.

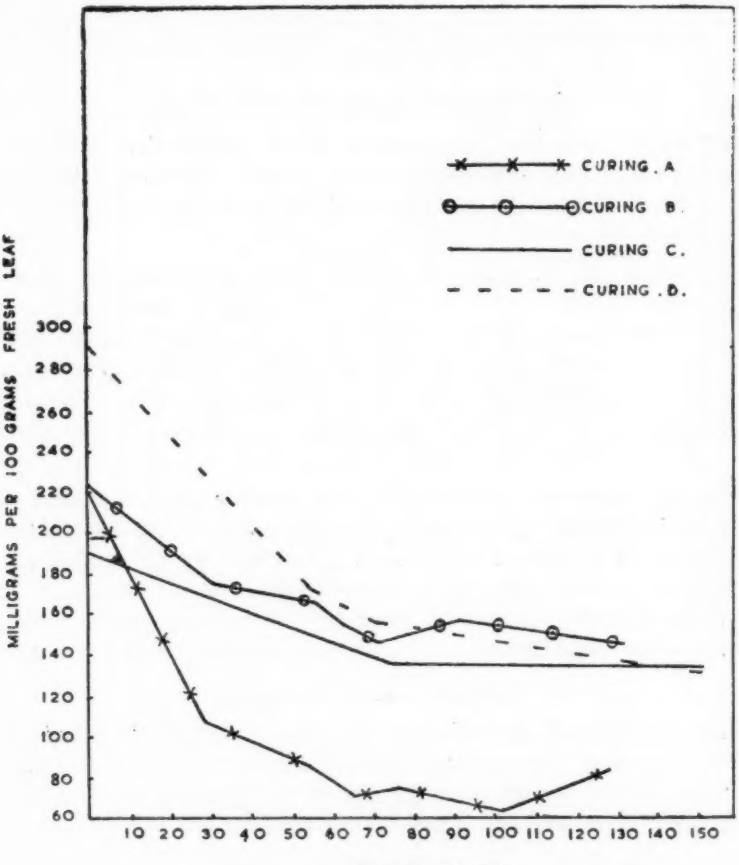


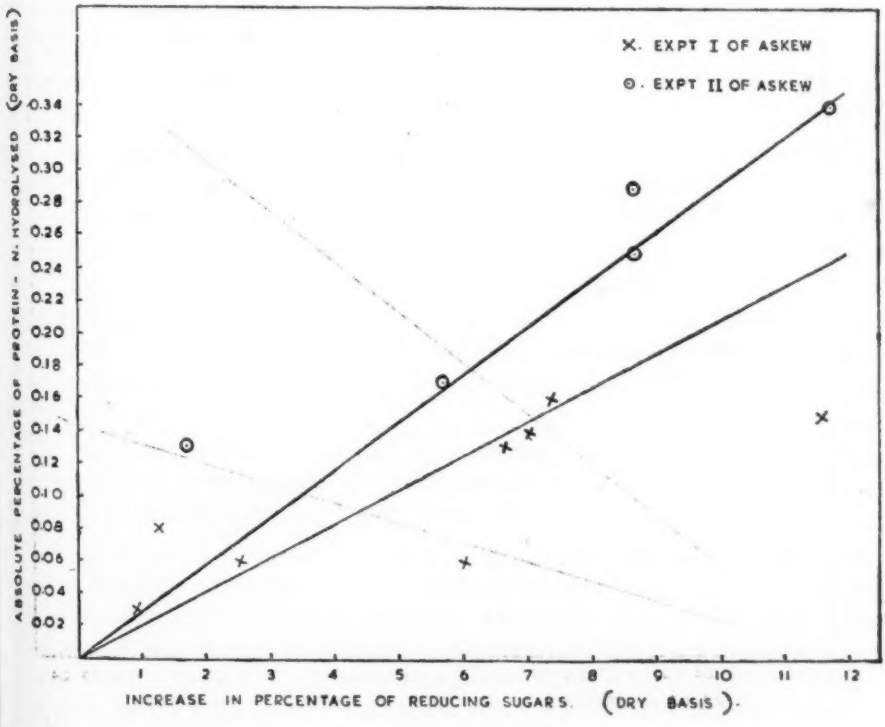
FIG. 1. Protein nitrogen in the leaf during flue-curing of tobacco.

Taking the changes in protein during yellowing only into consideration, it is obvious that though most of the protein hydrolysis took place during this phase in all the curings, there were sharp differences in the rates of hydrolysis in the different curings. From the slopes of the curves it is clear that the rate of proteolysis was the greatest in curings A and D while in curings C

and B it was much smaller. What is the factor which is responsible for these differences in the rate of hydrolysis of protein ?

Askew[1] plotted the increases in reducing sugars against the absolute percentage of protein, nitrogen hydrolysed during flue-curing and stated that a direct proportionality existed between these two. When the higher temperatures were reached however, the points were scattered due apparently to some reactions which resulted in reductions in sugars and hydrolysed nitrogen.

The plot of Askew's data, taking only the low temperature phases of flue-curing is presented in Fig. 2. When the first 55 hours of Experiment I of Askew are taken into consideration the line passing through the greatest number of points and origin covers only five points and three points are distinctly outside the line.



B2

FIG. 2

In the case of Experiment II the plot of data during the first 65½ hours is given and it shows that only 3 points are on the line while two are outside.

When the author's data[2] up to the end of fixing from curings A and B are plotted (Fig. 3) it is observed that one point definitely in curing A (2nd primings) and two points possibly in curing B (3rd primings) are outside the line. The number of points in the low temperature phases of curings C (6th primings) and D (7th primings) were too few to be plotted this way.

Thus there seem to be some instances where the relationship observed by Askew[1] does not appear to hold good.

Askew *et al.*[1] further observed that the greater rate of hydrolysis of protein in their leaves of Experiment II could be related to the more extensive hydrolysis of starch and greater loss of dry matter in that trial. It is interesting to see if these relationships are obtained in the author's data.[2] The

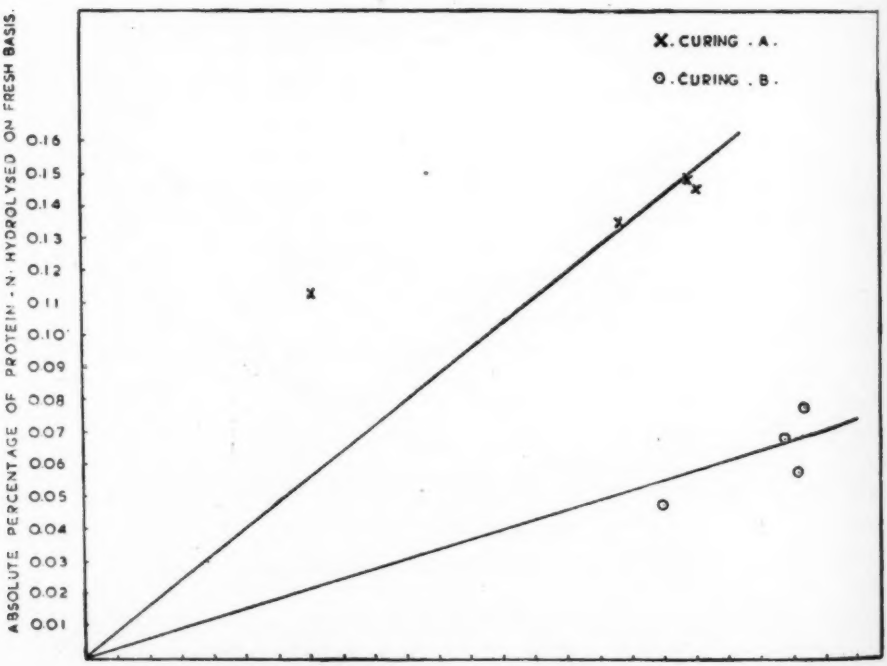


FIG. 3

relevant data extracted from the author's previous work[2] are presented in Table I.

TABLE I

Relationships between hydrolysis of starch, loss of dry matter and protein hydrolysis during flue-curing

Particulars of curing	Starch hydrolysed % original	Rate of hydrolysis of starch during yellowing m.gr./hour	Rate of loss of dry matter during yellowing m.gr./hour	Rate of hydrolysis of protein nitrogen during yellowing m.gr./hour
Curing A ..	75·8	80·0	25·2	2·50
Curing B ..	80·8	68·7	31·5	1·05
Curing C ..	75·0	67·6	34·8	0·74
Curing D ..	90·1	49·1	32·0	2·24

The above data appear to suggest that the rate of protein hydrolysis during yellowing in these leaves does not depend entirely on the rate of hydrolysis of starch or loss of solids or even on the amount of starch hydrolysed expressed as a percentage of the original.

The mechanism of protein synthesis in the plants is but incompletely understood. According to one view supported by Wood and co-workers[5–8] and Vickery and co-workers[9] proteins and amino-acids are in equilibrium with one another and protein synthesis takes place by step-by-step condensation of amino-acids.

The alternate view that protein synthesis and proteolysis are two distinct processes, not merely aspects of a reversible system, is supported by Gregory and Sen[10] and Steward and co-workers.[11–14]

According to the first view, if proteins and amino-acids are in equilibrium with one another, then the amount of protein nitrogen in the leaf, at the time it is harvested from the plant, represents the forces of synthesis and the amount of soluble nitrogen excluding nicotine nitrogen (nicotine does not appear to play any part in these reactions) the forces of hydrolysis. A ratio of these two is a measure of the factors that keep a certain level of protein in the attached leaf.

According to the alternate view advanced by Steward *et al.*[11–14] protein arises directly from sugar (or sugar derivatives) and ammonia rather than that the individual amino-acids are separately synthesized and then condense *via* peptides to proteins. If this were the mechanism of protein synthesis then again the amount of protein nitrogen in the leaves represents the forces of synthesis while ammonia multiplied by reducing sugars the forces of hydrolysis. The ratio of these two again gives a measure of the factors that keep a certain level of protein in the attached leaf.

These two ratios and the rate of protein hydrolysis are given in Table II.

TABLE II

Relationships of certain ratios and rate of protein hydrolysis during flue-curing

Particulars of curing	(Ratio I) Protein N / Ammonia N × reducing sugars	(Ratio II) Protein N / Soluble N—nicotine N	Rate of protein hydrolysis (m.gr./hour) on fresh basis
Curing A ..	0·19	2·18	2·50
Curing B ..	0·16	1·40	1·05
Curing C ..	0·09	1·62	0·74
Curing D ..	0·19	3·17	2·24

When these ratios are high the factors favourable for protein synthesis are predominant and when they are low factors favourable for hydrolysis are predominant in the attached leaf. When the leaf is detached from the plant it appears that these self-same factors work in the reverse direction and cause more rapid hydrolysis of protein when these ratios are high and less when they are low.

It is not claimed that these data throw any light on the path of protein synthesis. However, it is necessary to point out that the first ratio appears to more correctly indicate the rate of proteolysis than the second.

A search of literature revealed two sets of data which could be treated in a similar fashion. One is that of Askew *et al.*[1] and the other that of Vickery *et al.*[15–16] The first ratio, *i.e.*, protein N to the product of ammonia N and reducing sugars, could not be calculated from their data as some figures

were not available. The ratio of protein N to soluble N—nicotine N and the rate of proteolysis during the earlier phases of curing expressed as the amount of protein N (in 100 gr. fresh leaf) hydrolysed per hour are presented in Table III.

TABLE III

The rates of protein N to soluble N minus *nicotine N and rates of proteolysis during flue-curing and air-curing*

Particulars	Protein N	Rate of protein hydrolysis (m.gr./hour in 100 gr. fresh leaf)	Remarks
	Soluble N—Nicotine N		
Askew's leaves[1] of Expt. I (first 48 hours)	1·57	0·70	Flue-curing
Askew's leaves[1] of Expt. II (first 49½ hours)	3·91	1·11	
Vickery's leaves[15] 1931 (first 12 days)	2·11	0·61	Air-curing
Vickery's leaves[16] 1953 (first 12 days)	1·67	0·43	

In the case of Vickery's data the results relate to air-curing experiments conducted once in 1931 and again in 1953 using Connecticut shade-grown tobacco. Even so the higher rate of proteolysis in their leaves of 1931 is associated with a higher ratio. Similarly in the leaves of Askew *et al.*[1] which were flue-cured the higher rate of proteolysis in their leaves of Experiment II is associated with a higher ratio.

The slower rates of proteolysis in Vickery's leaves as compared with Askew's leaves are obviously due to the lower temperatures employed during air-curing of the former.

When the data in Tables II and III are taken in conjunction with the fact that conditions like temperature and humidity were different in the different curings, there appears to be a good reason to believe that the factors responsible for maintaining a certain protein level in the leaves when they are attached to the plant work in the opposite direction and cause its hydrolysis when they are detached from the plant.

SUMMARY

The rate of hydrolysis of protein during flue-curing was examined in the light of a relationship between protein and soluble nitrogen in the leaves at the time of harvest.

It was shown that a high ratio of protein N to (soluble N—nicotine N) is associated with a rapid rate of proteolysis and a low ratio with a slow rate.

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THE STRUCTURE OF THE SHOOT APEX AND LEAF INITIATION IN *POLYALTHIA LONGIFOLIA*

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In recent times, the order Ranales as broadly conceived by Engler, is attaining an important role in discussions pertaining to phylogeny of angiosperms. Efforts toward this end have been initiated by the Harvard school of botanists headed by Prof. Bailey and through their publications much valuable information in regard to the comparative morphology of vegetative structures has become available. Morphogenetic aspects of the representatives of this order, however, are yet to be investigated and determined, which in turn should be harmonised with data obtained through other botanical disciplines. The present study was undertaken to add additional information regarding the structure of the shoot apex and leaf differentiation in a woody ranalean member.

The study pertaining to the structure of the shoot apex and leaf initiation in seed plants is receiving much importance during the past few decades. Amongst these studies, some of the ranalean representatives have also been included: Winteraceæ, Schisandraceæ, Illiciaceæ, Trochodendraceæ, Tetracentraceæ (Gifford, 1950, 1951 and 1954) ; Magnoliaceæ (Millington and Gunkel, 1950) ; Lauraceæ (Kasapligil, 1951) and Ranunculaceæ (Tepfer, 1953).

These investigations, although of an exploratory nature, indicate broadly that (*a*) fluctuation exists in regard to the dimensions and shape of the shoot apex not only amongst the different genera but also perhaps within the same species, and that (*b*) the number of tunica layers is generally two, occasionally three, this order of variation sometimes being present within the same species or very likely within the same tree. As a detailed analysis of the results obtained from the studies of shoot apices has been recently reviewed by Gifford (1954), the discussion is kept to a minimum in the present study. It may also be stated in this connection that the terminologies as proposed by Schmidt (1924) have been employed in this paper to designate the cell layers of the shoot apex.

MATERIALS AND METHODS

The materials were collected fortnightly during successive seasons of the vegetative growth phase from carefully selected trees growing in the vicinity. Actively growing vegetative buds and embryonic leaves in various stages of development were fixed in FAA, dehydrated and processed through customary methods. Sections were cut 8–10 μ thick and stained in hæmatoxylin-erythrosin combination.

OBSERVATIONS

At the maximal phase of plastochron, the shoot apex appears more or less dome-shaped, the side adjacent to the youngest leaf primordium being always less taller than all around (Fig. 10). At such a stage there are two uninterrupted tunica layers (T 1 and T 2) and a subjacent core of corpus cells. The outer tunica layer is composed of more or less isodiametric cells, while the cells of the inner layer exhibit slight anticlinal elongation (Fig. 10). The two tunica layers together maintain their identity through repeated divisions in anticlinal plane. Only, in the second tunica layer, periclinal divisions occur during leaf initiation (Fig. 10). The corpus is made up of a mass of highly meristematic cells, more or less isodiametric in their shape and this central mass of corpus corresponds to the mother-cell-zone. A radiating zone in the corpus is absent. Lying subjacent to corpus is a rather restricted and incipiently differentiated pith-rib-meristem composed of nearly vacuolated cells extending to a few cell layers where it matures into the parenchymatous pith below (Fig. 10). A group of three to four cells present on the extreme summit of the apex in the outer tunica layer (T 1) and two or three cells laying just below it in the second tunica (T 2) stain less deeply as compared with the neighbouring cells on all sides (Fig. 10). This corresponds to the vacuolated zone and is restricted to the tunica layers only.

The width of the apex during the maximal phase of plastochron (*i.e.* that phase of the vegetative apex wherein the cells in the second tunica layer have just divided periclinally to form the locus of leaf initiation) is 68 μ and the height of the apex measured from the base of the corpus is 44 μ. A procambial strand differentiating acropetally was noticed to be present 76 μ short of the apex (Fig. 3). The free end of this strand was often found to be associated with the locus of initiation of a leaf primordium. However, in a majority of cases, the initiation of a leaf primordium preceded the differentiation of the procambial strand supplying the corresponding leaf.

At the minimal phase of plastochron, the width of the apex is 43 μ and the height measured from the base of the corpus is 28 μ. At this phase the shoot apex is associated with an actively growing leaf primordium. Due to the rapidity in the growth of the leaf primordium, the shoot apex becomes distorted in shape ; the surface becomes almost flat and slanting in relation to the vertical axis (Figs. 1, 2 and 14). The tunica generally remains two-seriate and the constituent cells retain the same shape as at the maximal phase of plastochron (Compare Figs. 10 and 13). Conspicuous vacuolation in these cells is absent. The corpus is composed of isodiametric cells and lies subjacent to the tunica. At the minimal phase, numerous divisions in anticlinal plane in the tunica layers and in diverse planes in the corpus cells occur in the apex in expectation of the successive appendicular structures. Even though the second tunica layer exhibited anticlinal divisions during the resolution of the apex to the maximal phase, it was not uncommon to observe periclinal divisions in the same (arrow in Fig.13), the derivatives of which are added to the corpus zone. The outer tunica layer (T 1) always divides anticlinally perpetuating itself and contributes to the protoderm.

Sometimes the peripheral cells of the corpus appear to align themselves (Fig. 11) so as to simulate a layer of tunica, the constituent cells having undergone slight anticlinal elongation. Such a condition introduces the possibility of interpreting the shoot apex as possessing three layers of tunica. However, since two tunica layers are present as a general case, the third layer, when present, may alternatively be considered to be a particular manifestation of the corpus cells.

A comparison of the shoot apex at the maximal phase with that at the minimal phase will reveal vast differences that have occurred in the apex with the inception and subsequent growth of the leaf primordium. The more or less dome-shaped apex of the maximal phase (Fig. 10) becomes reduced in size, shape, width and height during the minimal phase and is represented as a slightly raised structure at the base of the vigorously growing primordium (Figs. 2, 13 and 14). In other words, with the inception of a foliar primordium, the growth activity in the corpus region seems to become largely directed towards the developing primordium.

Initiation of Leaf

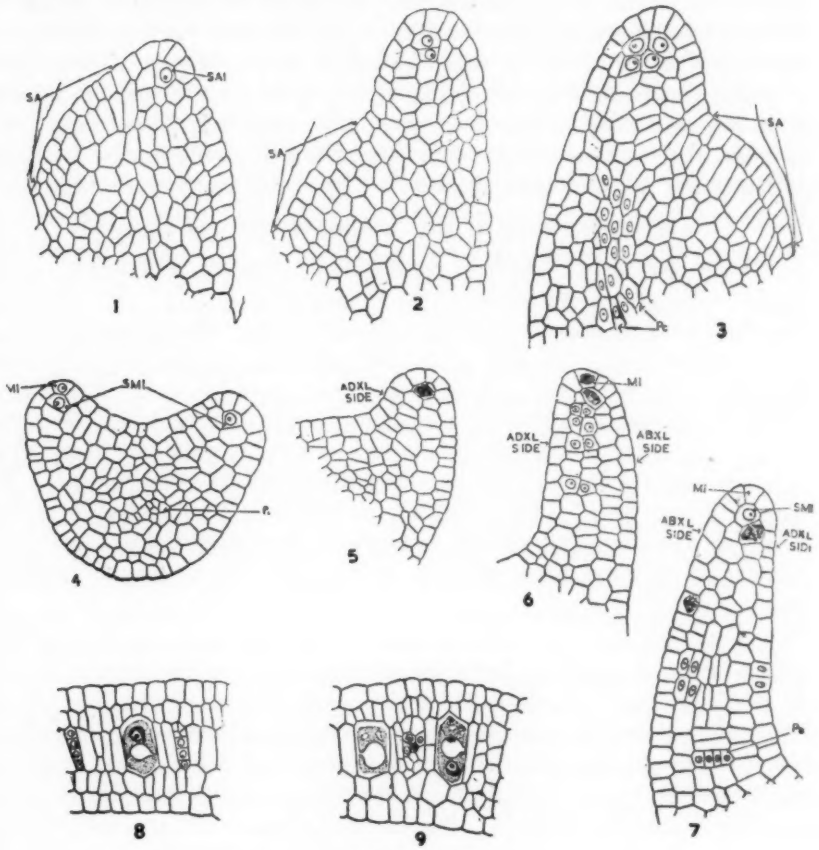
The leaves are initiated alternately over the apex establishing the characteristic phyllotaxy of the adult plant which is distichous or bifarious. This situation becomes manifest in young shoots by the zig-zag arrangement of the successive internodes. Such a nature of the stem becomes established in the early stages of foliar ontogeny. With the initiation and subsequent

growth of each foliar primordium, the shoot apex becomes deflected from the erect to the slanting position, and further growth of the apex in the next plastochron proceeds *in situ* (*see* Figs. 2, 3, 11, 13 and 14 wherein the apex is deflected from its erect position).

During the initiation of a leaf primordium, generally two to three (less frequently one) adjacently placed elongated cells of the second tunica layer, all situated more or less in the region of the shoulder of the apex, divide periclinally (arrow in Fig. 10). This division signifies the origin of a leaf primordium. Following this division there is a slight swelling at the corresponding surface of the apex and this constitutes the foliar buttress. Concomitant with the initiation of the foliar buttress, the cells in the subjacent region of the apex tend to divide, mostly in periclinal plane, so that the resulting cells enlarge and aid in the initial heightening of the primordium above the level of apex. Thus, initial heightening of the primordium is brought about by the activity of the corpus cells at the base of the foliar buttress. Further heightening of the primoridum is taken over by the activity of the sub-apical-initial cell which becomes established as soon as a definable primordium is initiated over the apex (Fig. 1). A series of cell divisions occur in this cell and in its derivatives. Often however, the four cells that result from periclinal division in the two adjacent cells in T-2 layer at the time of leaf initiation, occupy the locus of a single sub-apical-initial cell and function in a like manner (Figs. 3 and 14). This condition becomes manifest in primordia 46 μ high. However, in older primordia the single sub-apical-initial cell is of common occurrence. Hence it can be seen that for a time at least, the growth of the primordium in the vertical direction is largely due to the activity of a group of sub-apical cells. When such a group functions, the derivatives tend to arrange themselves in a radiating manner from the two tiers of sub-apical-cell group (Figs. 3 and 14). Figs. 12 and 15 show respectively an anticlinal and a periclinal division in a sub-apical cell of a leaf primordium. These two photographs are taken from alternate sections of the same leaf primordium within a distance of 10 μ.

The sub-apical-initial cell divides anticlinally (Fig. 12) and periclinally (Figs. 2, 13 and 15) contributing cells to the sides and central core of the primordium respectively. From this situation it should not be inferred that the apical extension of the primordium is largley due to the activity of the sub-apical-initial cell alone. In addition to the apical growth, there is also an active intercalary growth in the primordium, during which the divisions take place mostly in periclinal planes while anticlinal divisions are

also of frequent occurrence. An examination of Fig. 15 will reveal three periclinal achromatic figures in various stages of division, apart from the one in the sub-apical-initial cell proper, as also an anticlinal division in one of the subjacent cells (all indicated by arrows). Apical growth in a leaf primordium ceases when it is about 770 μ high.



TEXT-FIGS. 1–9

Procambial differentiation in a young primordium becomes discernible when the latter is about 48 μ high (Fig. 3). The cells are distinguished by their richly stainable protoplasts, elongated shape and large nuclei. The course of differentiation is always acropetal, starting in continuation of the procambial traces of the stem part. Generally, when there is apical growth occurring in a primordium, procambial differentiation does not proceed

up to the tip of the primordium (Fig. 12). In such cases, the derivatives of the sub-apical-initial cell arrange themselves in the path of the procambial development. An examination of Fig. 13 will reveal the orderly seriated manner in which the derivatives of the sub-apical-initial cell become arranged. These cells differentiate later as procambial cells in continuation with the subjacent procambial strand (Figs. 12 and 15). The derivative cells are at first isodiametric just like any other neighbouring cell, and mature directly into procambial cells assuming the histological characteristics. These trends of events clearly indicate that the development of the procambium proceeds from base upwards in a gradual manner and not simultaneously, and, also that the derivatives of the sub-apical-initial cell between the latter and differentiated procambium directly mature into procambial cells.

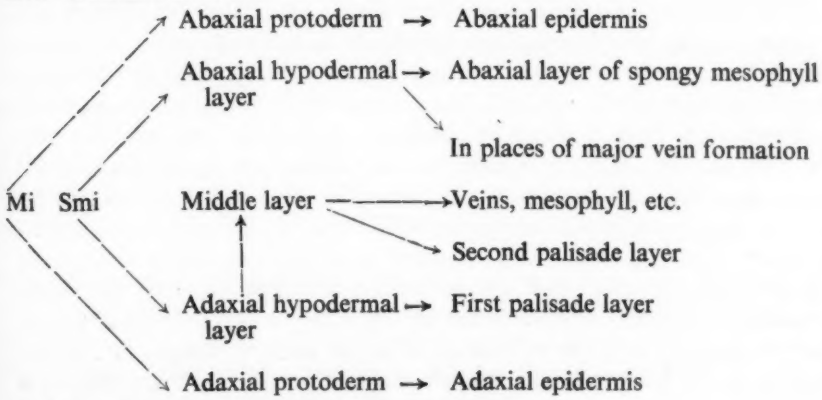
MARGINAL GROWTH AND DIFFERENTIATION OF LAMINA

When the petiolar-midrib procambium becomes discernible in the median part of the primordium, marginal growth sets in. At this phase, the primordium, as seen in transection, presents a heart-shaped outline (Fig. 4). The sub-marginal-initial cell is located adaxially in the elevated flanks (Fig. 4). The initial cells are relatively more conspicuous by their slightly enlarged size and more pronounced polygonal outline (Fig. 4). The outer cell that abuts the sub-marginal-initial cell is the marginal-initial cell which is responsible for the increase in the surface area of the epidermis. The marginal-initial cell divides essentially in anticlinal planes (Fig. 6) contributing derivatives to the abaxial and adaxial protoderms, which by further divisions in anticlinal plane give rise to the epidermis of the corresponding side.

On the contrary, the entire internal tissue organisation of the leaf—the palisade, the mesophyll, the veins, the oil cells, etc., owes its origin to the activity of the sub-marginal-initial (hereafter designated as *smi*) cell and its derivatives. To begin with, the *smi* always divides anticlinally and contributes derivatives to the abaxial and adaxial hypodermal layers (Figs. 5 and 6). The derivatives to the abaxial side of the lamina align themselves in an undisturbed regular row (Fig. 6). The plane of division in these cells is mostly anticlinal. Periclinal divisions occur in one or two adjacent cells of this layer (Fig. 7) and these divisions are associated with the development of major vein of the lamina. On the contrary, the derivative cells of the adaxial side behave quite differently. These cells divide either periclinally (Fig. 6) or obliquely (Fig. 7) to produce derivatives to the middle layer. Thus, the middle layer of cells is almost exclusively derived from the adaxial hypodermal layer. Such a derivation of the middle layer wholly from the adaxial layer is a condition that appears to be rather infrequent, and has been reported

by Gifford (1951) in *Drimys winterii* and by Mericle (1950) in *Zea*. The differentiation of the middle layer from the adaxial side may be accomplished generally immediately after a cell is derived from the *smi* cell to the adaxial layer (Figs. 6 and 7) or occasionally at a slightly later stage.

When the embryonic lamina consisting basically of five layers of cells becomes determined, the middle layer elongates considerably (Fig. 8) thereby contributing to the increase in the thickness of the leaf. By this time the adaxial hypodermal layer tends to elongate in order to assume the conventional form of the palisade cells. Further tissue differentiation proceeds in the middle layer of cells, the first kind to differentiate being the procambium of the vein categories. Whenever a single cell is involved in the initiation of a vein, the divisions that occur in the cell are such as to produce a tier of cells (Fig. 8). If, on the other hand, more than one adjacently lying cells should initiate vein development, the divisions occur in diverse planes so that a nest of small cells is produced (Fig. 9). These form the procambial cells of the future vein system of the leaf. Later, the middle layer in its course of further tissue differentiation, cuts off a regular row of cells towards the adaxial part of the lamina. These cells arrange themselves underneath the elongating adaxial hypodermal layer to differentiate and constitute the future second palisade layer of the leaf. Thus, it will be seen that the two palisade layer of the mature leaf, even though morphologically and functionally are similar, owe their origin to different cell layers of the embryonic lamina. In places of major vein development, the second and at times the first palisade layer is either reduced in its size or becomes suppressed. The following figure shows the activity of the marginal-initial and *smi* cell and their derivatives.


Abaxial protoderm → Abaxial epidermis
Abaxial hypodermal layer → Abaxial layer of spongy mesophyll
In places of major vein formation
Mi
Smi
Middle layer → Veins, mesophyll, etc.
Second palisade layer
Adaxial hypodermal layer → First palisade layer
Adaxial protoderm → Adaxial epidermis

Mi—Marginal initial cell; Smi—Sub-marginal-initial cell.

DISTRIBUTION AND DIFFERENTIATION OF THE OIL CELLS

In the mature leaf, the oil cells or the secretory idioblasts (Foster, 1956) are distributed in the spongy mesophyll and palisade of the leaf. Those cells of the embryonic lamina which are destined to differentiate as the oil cells can be distinguished from others of the layer by their enlarged size, dense cytoplasm, enlarged nucleus and avidity for cytoplasmic and nuclear stains. Such a cell gradually increases in size and simultaneously with this a vacoule lined with a thin pellicle begins to enlarge in one corner of the cell (Figs. 8 and 9). Subsequent enlargement of the vacuole causes it to occupy almost the entire lumen of the cell, whereby the protoplast is pushed against the cell-wall. It should be pointed out here that the oil cells in the spongy mesophyll of the leaf differentiate somewhat simultaneously whereas those in the palisade differentiate rather late. Thus the differentiation of these cells in the tissues of the leaf is spread over a longer period in time. Often when the oil cells develop in the second palisade layer, the adjoining similar cells and the juxtaposed cells of the other palisade layer lose their conventional elongated form and undergo such changes in shape as determined by the enlargement of the oil cell in question.

DISCUSSION

The present investigation reveals that the shoot apex of *Polyalthia longifolia* as in many other investigated angiosperms (*see* Gifford, 1954 and literature cited therein) consists of an uninterrupted biseriate tunica and a central zone of corpus. In a majority of cases, corpus is said to exhibit a zonate pattern (refer to the review by Gifford, 1954), which is based upon the seriated manner of cell arrangement in the corpus. In the shoot apices of *Polyalthia*, however, such a zonation is not evident in the corpus.

The vacuolation of certain cell groups of tunica and corpus appear to characterise a large number of shoot apices in angiosperms. Amongst Ranales its presence is reported in *Drimys*, *Trochodendron*, *Tetracentron*, *Pseudowintera*, *Illicium*, *Kadsura* (Gifford, 1950) ; in *Umbellularia* and *Laurus* (Kasapligil, 1951) ; in *Liriodendron* (Millington and Gunkel, 1950) and in *Aquiligia* and *Ranunculus* (Tepfer, 1953) and as published illustrations are concerned, this feature appears to be well established in the vesselless genera (Gifford, 1950, 1951). Similar vacuolation of certain cell groups in tunica and corpus is well evident in the vesselless species *Sarcandra irvingbaileyi* (unpublished observation of the author). In *Polyalthia*, vacuolation is restricted to a few cells situated in the extreme summit in the two tunica layers of the apical dome and the corpus does not show any definable vacuolation.

The occurrence of fluctuation in the number of tunica layers is reported in the shoot apices of many angiosperms (for complete citations *see* Gifford, 1954). The stable number of tunica layers generally seems to be two in angiosperms. This condition seems to be well preserved in the Ranales except in the vesselless genus *Tetracentron* wherein the second tunica layer seems to be ill-defined (Gifford, 1950). *Notophoebe* (Lauraceæ) seems to exhibit a stabilised number of three tunica layers (Kasapligil, 1951). The third tunica layer spoken of by Millington and Gunkel (1950, Fig. 9) in *Liriodendron* seems to the present writer to be only a stratification of the corpus cells, since the vegetative apex depicted in the concerned illustration is associated with an actively growing buttress of a foliar or stipular appendage. At such a phase it is but natural to expect numerous cellular divisions in the apex in order to accommodate the actively growing buttress and the apex to enlarge so as to accommodate the next foliar structures. Hence, at such a phase, it is quite possible for the corpus cells that are subjacent to tunica to become stratified into an alignment simulating a tunica layer as a provisional measure. Similarly, Gifford (1950) reports a stratification of corpus cells resembling the tunica layers in the active shoot apices of *Drimys winterii* and this condition is said to be lost as soon as the leaf is initiated over the apex. In *Polyalthia* it has been shown in the observation part that the superficial cells of the corpus stratify into a layer resembling tunica. In this plant, this situation seems to be achieved while the apex reorganises from the minimal to the maximal phase.

Amongst the investigated ranalean genera, the maximum dimension in width is exhibited in *Kadsura*, the apex measuring 262 μ in width and 62 μ in height. In *Polyalthia*, the apex measured at the maximal phase of plastochron is 68 μ wide and 44 μ high. Taking into consideration the fluctuation that may exist in such measurements (refer Gifford, 1954, pp. 498–499) it is certain that the apex in *Polyalthia* exhibits the least dimensions amongst the so far investigated ranalean genera both in width and height. Further, the shape of the apex varies in ranalean genera from that of a flat nature in *Drimys* to that of a hemisphere in *Trochodendron* (Gifford, 1950). The shape of the apex in *Polyalthia* more nearly approaches the condition in *Trochodendron* but with asymmetrical sides.

No seasonal variation affecting the shoot apices in *Polyalthia* was noticed. Shoot apices from collections made throughout a year denote no change in the fundamental pattern of the apex. Periclinal divisions occur in the second tunica layer augmenting the formation of the foliar buttress. This course of foliar initiation seems to be universal to many dicotyledonous

members without any relation to the number of tunica layers which generally varies from one (Boke, 1941, 1951, 1952, 1953 ; Esau, 1942 ; Phillipson, 1947 ; Ball, 1941 and others) to as many as four (Cross, 1937 *a*; Zimmermann, 1928). In cases where more than two layers of tunica are reported, the participation of the other layers of tunica varies as regards their contribution to the developing leaf primordium (*cf.* Cross, 1937 *a*, *b* ; Foster, 1935 *a*, *b* ; Boke, 1940). According to Esau (1953), the fewer the number of tunica layers, the more is the contribution of corpus to the developing leaf primordium. In *Polyalthia*, the tunica layers are two in number and hence it would ordinarily be expected that the corpus derivatives would aid in the initial heightening of the primordium. But it is observed that the corpus does not contribute to the primordium and at best the divisions in the cells of the corpus heightens the primordium above the level of the shoot apex only. The early establishment of the sub-apical-initial cell in the primordium restricts the amount of contribution by the corpus cells and the heightening of the primordium is carried over by this cell. Similar early establishment of the sub-apical-initial cell in foliar ontogeny has already been reported in the phyllode primordium of *Acacia* (Boke, 1940), in the cataphyll primordium of *Carya* (Foster, 1935 *a*) and in the foliar primordium of *Linum usitatissimum* (Girolami, 1954). On the contrary, a different situation is reported in the vesselless plant *Drimys winterii* (Gifford, 1951) wherein the sub-apical-intial cell of the foliar primordium is delayed until the primordium is 80 μ high and this is said to be characteristic of leaves produced during late summer and fall. Further, corpus derivatives are said to be present in the primordium of *Drimys* up to a height of 90 μ.

Instances are not lacking wherein the early heightening of the primordium is attributed to a group of sub-apical cells rather than to a single sub-apical-initial cell. The possible presence of a group of four sub-apical cells is indicated in *Acacia* (Boke, 1940), in *Carya* (Foster, 1935 *a*) and a group of six sub-apical cells in the foliar primordium of *Drimys* (Gifford, 1951). Early growth of the primordium apically is attributed to this group of sub-apical cells (Boke, 1940 ; Foster, 1935 *a*). Similarly, in *Polyalthia* is present a group of sub-apical cells which augment the heightening of the primordium especially of an height of 46 μ. Added to apical growth by the activity of sub-apical cell there is an active intercalary growth in the cells of the primordium which also aid in the heightening of the appendage.

In general, apical growth activity in the foliar primordium of dicotyledons ceases late in ontogeny than in either monocotyledons or gymnosperms. In *Nicotiana tabacum* (Avery, 1933) apical growth ceases when the primordium

is 2–3 mm. in height ; in *Viburnum* (Cross, 1937 *a*) apical growth continues till the primordium attains a height of 1,600 μ ; in *Drimys* (Gifford, 1951), it can be traced in primordia as high as 1,148 μ ; in *Acacia* (Boke, 1940) apical growth is seen until the primordium attains 500 μ in length. Hence the cessation of apical growth activity in the foliar primordium of *Polyalthia* at a height of 770 μ lies at the lower range of variability for dicotyledons.

The reviews of Foster (1936, 1949) and Esau (1953) describe two main types of sub-marginal-initial cell activity during the marginal growth of the lamina. In these, the middle layer or core of cells of the embryonic lamina (as viewed in transection), which yield to the further tissues of the leaf, is formed from the *smi* cell directly (Avery, 1933; Cross, 1937 *a*; Gifford, 1951, etc.) ; or the middle core is originated due to the activity of the abaxial layer (Cross, 1936, 1937 *b* ; Foster, 1935 *a*, 1936 ; Girolami, 1954, etc.). Deviation from these two modes of developmental pattern of the middle layer has already been reported. These instances are fewer in number. In *Zea* (Mericle, 1950) the middle layer of cells is said to arise from the adaxial hypodermal layer of the embryonic lamina. So also in *Drimys* (Gifford, 1951), a similar condition, whereby the adaxial hypodermal layer contributes to the middle core, is reported. In *Drimys*, however, the central core of cells in the embryonic lamina is derived also due to the activity of the abaxial hypodermal layer and the *smi* cell. In *Liriodendron* (Pray, 1955) also, the middle layer is said to arise in part from the adaxial hypodermal layer. The condition in *Polyalthia* seems to be unique in that the middle layer of cells of the embryonic lamina is almost exclusively derived from the adaxial hypodermal layer, the abaxial hypodermal layer contributing derivatives only in places of major vein development. Though the above mode of marginal meristematic activity is infrequently reported at present, it should not be construed as an uncommon occurrence in the dicotyledons.

The mature leaf in *Polyalthia* contains two layers of palisade cells conforming to the conventional shape. Even though these two layers resemble each other morphologically and functionally, yet they are derived from different layers of the embryonic lamina. The adaxial layer of cells, after giving rise to the middle layer, elongate to form the first palisade layer while the second palisade layer is derived from the middle layer in its course of tissue differentiation. A parallel origin of the two palisade layers is reported in *Viburnum* (Cross, 1938).

Procambium is seen to differentiate acropetally in the stem apex and leaf primordium as in other plants.

SUMMARY

The shoot apex in *Polyalthia longifolia* consists of two tunica layers uniformly present all through a year, and a homogeneous mass of corpus cells. Corpus does not exhibit any zonate pattern and is composed of a mass of isodiametric cells. The rib-meristem is incipiently differentiated and lies subjacent to the corpus. Stratification of the outer corpus cells resembling second tunica layer is noticed to occur while the apex reorganises from minimal to maximal phase. The second tunica layer exhibits anticlinal elongation while the cells of the outer tunica layer is more or less isodiametric in shape. A group of cells of the tunica layers at the extreme summit of the apex exhibit conspicuous vacuolation. *Polyalthia longifolia* appears to possess the smallest size of shoot apex amongst the ranalean families so far investigated.

The formation of the foliar buttress is preceded by periclinal divisions in the cells of the second tunica layer. The contribution of the corpus derivatives to the foliar primordium is very much restricted and the sub-apical-initial cell is established as soon as a definable primordium becomes apparent on the apex. The presence of a group of four sub-apical cells is indicated in young primordia. Apical growth of the leaf primordium ceases when it attains a height of 770 μ. The developing leaf primordium alters the orientation of the shoot apex, whereby the stem attains a corresponding zig-zag contour.

Materials sectioned from collection made during different seasons of a year denote no change in the fundamental pattern of the apex.

During marginal growth of the leaf primordium, the adaxial hypodermal layer gives rise exclusively to the middle layer of cells, while the abaxial hypodermal layer contributes occasionally in loci of major vein development. The distribution and differentiation of oil cells in leaves is traced. Procambial differentiation is acropetal in the shoot apex and the leaf.

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I take this opportunity to express my gratitude to Professor B. G. L. Swamy for guidance and helpful criticisms throughout the course of this investigation.

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EXPLANATION OF TEXT-FIGURES

FIGS. 1–9. Fig. 1. Median longitudinal section of the shoot apex with an emerging leaf primordium. The presence of the sub-apical-initial cell can be noticed in the primordium, ×375. SAI—sub-apical-initial cell; SA—denotes the limit of the shoot apex. Fig. 2. Median longitudinal section of a leaf primordium showing the periclinal division of the sub-apical-initial cell, ×375. SA—denotes the limit of the shoot apex. Fig. 3. Median longitudinal section of the shoot apex with a leaf primordium. A group of four sub-apical cells can be seen in the primordium, ×375. SA—denotes the limit of the shoot apex; Pc—procambial cells; Fig. 4. Transverse sectional view of a young primordium (at the time of marginal growth), ×375. SMI—sub-marginal-initial cell; MI—marginal initial cell; Pc—procambial cells. Fig. 5. Transverse sectional view of a young primordium showing anticlinal division in the sub-marginal-initial cell, ×375. ADXL side—adaxial side of the lamina. Fig. 6. Transverse section of an embryonic lamina. Note the anticlinal division of the sub-marginal-initial cell and periclinal division in the cells of the adaxial hypodermal layer, ×375. MI—marginal initial cell, ABXL side—abaxial side; ADXL side—adaxial side. Fig. 7. Transverse section of an embryonic lamina. Note the periclinal division of the cells of the abaxial layer in places of major vein development, division of the cells of the middle layer to form a tier of procambial cells and oblique division of the adaxial hypodermal layer contributing derivative to the middle layer, ×375. MI—marginal initial cell; SMI—sub-marginal-initial cell; ABXL side—abaxial side; ADXL side—adaxial side; Pc—procambial cells. Figs. 8 and 9. Transverse sections of embryonic lamina showing the differentiation of the oil cell. Also note the tiered and grouped arrangement of the procambial cells, ×375.

EXPLANATION OF PLATE X

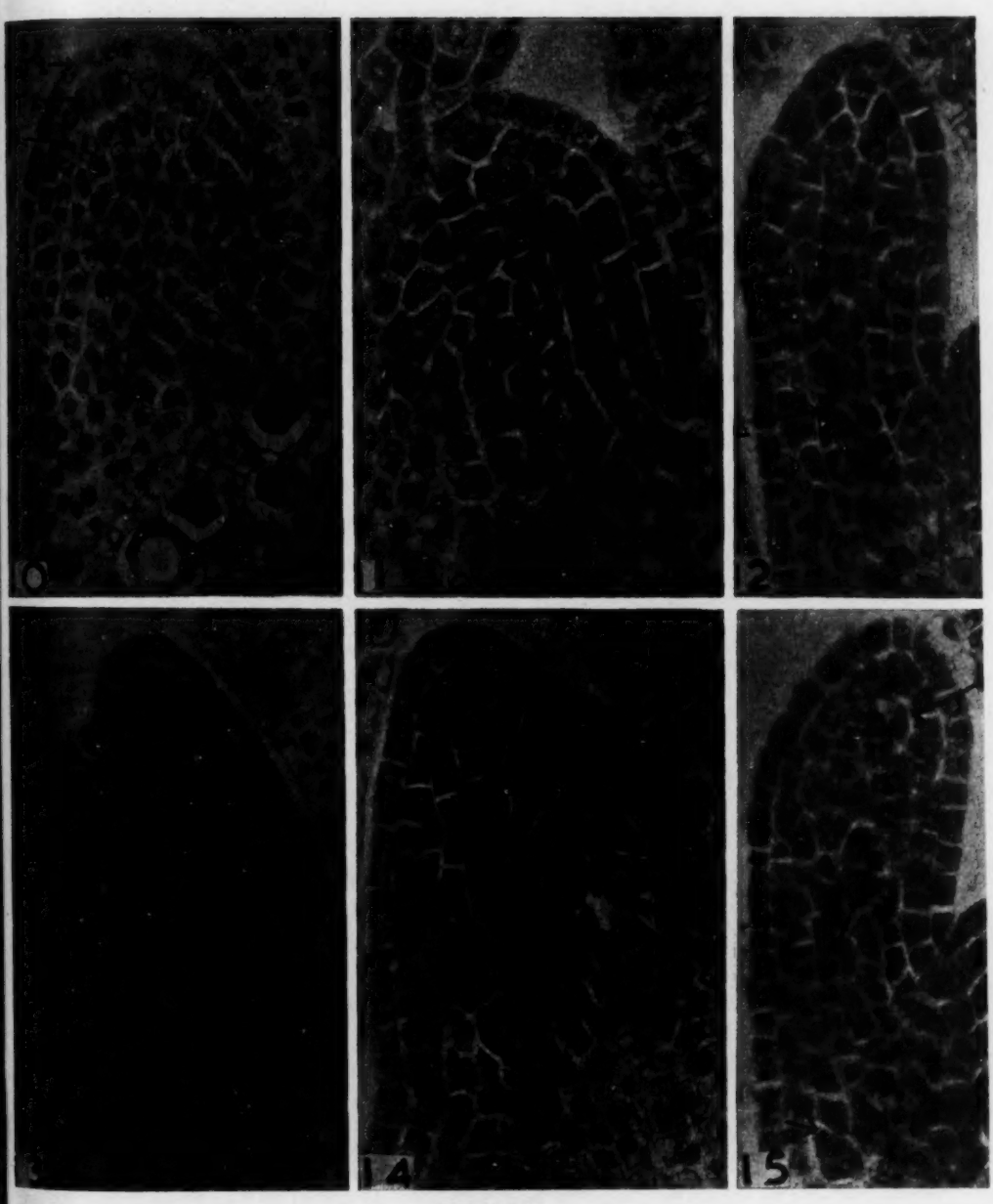
FIGS. 10–15

FIG. 10. Median longitudinal section of the shoot apex at maximal phase of plastochron. Note the vacuolated cell group restricted to the tunica layers at the extreme summit of the apex. Periclinal divisions in the second tunica layer can also be seen (indicated by arrow), ×530.

T 1—First layer of tunica.

T 2—Second layer of tunica.

P.d.—Periclinal division in the second tunica layer. The divisions indicate the locus of leaf initiation.



FIGS. 10–15

FIG. 11. Median longitudinal section of the shoot apex showing the stratification of the outer corpus cells resembling second tunica layer, ×610.

FIG. 12. Median longitudinal section of a leaf primordium showing anticlinal division in the sub-apical cell. Pc—Procambium, ×485.

FIG. 13. Median longitudinal section of the leaf primordium. The section is slightly peripheral with regard to the apex. Periclinal division (indicated by arrows) can be seen in the second tunica layer, the derivatives of which are added to the corpus. A row of four cells can be seen to radiate from the sub-apical-initial cell in the leaf primordium, ×425.

FIG. 14. Median longitudinal section of the leaf primordium, showing a group of four sub-apical cells, ×335.

FIG. 15. Longitudinal section of the leaf primordium showing the occurrence of periclinal division in the sub-apical cell. Arrows indicate the various achromatic figures responsible for intercalary growth, ×485.

INVESTIGATIONS ON THE FAILURE OF PEAS IN 'GORADU' SOILS OF GUJARAT

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Charotar area of Central Gujarat is considered to be one of the most fertile parts of Bombay State. The soil of this region known as 'Goradu' is a sandy loam with 81% sand and 10% clay. It is an ideal land for intensive cultivation under irrigation and grows a variety of crops, both leguminous and non-leguminous.

Several leguminous crops such as bean (*Dolichos lablab*), Cow pea (*Vigna catjeng*), *guar* (*Cyamopsis tetragonoloba*), gram (*Cicer arietinum*), etc., grow successfully in 'goradu' soils without the seeds having to be inoculated with the specific nodule bacteria. These legumes form abundant nodules in their roots indicating the presence in the soil of the specific nodule bacteria in sufficiently large numbers. But in the case of the garden pea (*Pisum sativum*) the growth of the plant is poor and there are only poorly developed nodules to be found in its roots indicating that the pea nodule bacteria do not exist in sufficient numbers in the soil. If, however, the pea seed is inoculated with the specific root nodule bacteria, *viz.*, *Rhizobium leguminosarum*, Frank, plenty of nodules are formed in the root of the plant and the crop grows successfully. The above observation raises the question regarding the ability or otherwise of pea rhizobia to occur normally in these soils, and several factors may account for this position. Soil is a complex medium where innumerable physical, biochemical and biological activities are constantly in progress and only careful investigations can lead to the understanding of the problem. In Broach and Surat Districts where peas are grown successfully without seed inoculation, the soil is black and sticky and has a high clay content and consequently a higher moisture retaining capacity whereas the 'goradu' soil of Gujarat has a high proportion of sand, and poor moisture retaining capacity. The nature of the 'goradu' soil perhaps associated with other factors was therefore considered unsuitable for the survival of *R. leguminosarum*. Some of these other factors might be the biochemical changes in the soil, presence in the soil

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of other microflora antagonastic to the bacteria, etc. It is however reasonable to assume that the pea nodule bacteria have physiological and biological properties different from those of other root nodule bacteria normally present in the soil. A comparative study of pea rhizobia with other groups of rhizobia present in the soil was therefore undertaken in this laboratory.

Sources of rhizobia under study.—Rhizobia were isolated from healthy root nodules of some common leguminous crop plants and weeds occurring in 'goradu' soils of Charotar. The crop plants selected were bean, guar, berseem (*Trifolium alexandrinum*), lucerne (*Medicago sativa*) and 'Shewri' (*Sesbania ægyptiaca*). It may be mentioned that berseem and lucerne grow well for a number of years on the plot after one inoculation of the seed. The weeds selected were *Zornia diphylla* and *Tephrosia purpurea*. Two pea cultures (Nos. 1 and 2) were secured from Agriculture College, Poona and No. 4 was a culture isolated from plants growing in the black soils of Broach. Isolate No. 3 was one obtained from the plots in 'gorad' area inoculated with the special culture the previous year.

Isolation of bacteria from the nodules was done by the usual methods, on yeast extract mannitol congo-red (1 : 20,000) and crystal violet (1 : 80,000) agar.

All the isolates were then tested for the absence of *Agrobacterium radiobacter*, on litmus milk, plain milk, potato slants, yeast extract mannitol broth pH 10 and pH 11–12, yeast extract mineral salts medium with $CaCO_3$, *plus* (*a*) 1% dextrin, (*b*) 1% inulin, Koser's uric acid medium, mannitol calcium, glycerophosphate agar, crystal violet glycerol nitrate soil extract agar, and for the production of H_2S (Vyas and Prasad, 1959). All the isolates were found to be free from *A. radiobacter*. The isolates were also tested for their ability to produce nodules on the respective host plants under aseptic conditions (Vyas, 1955).

Effect of soil fungi on rhizobia.—Presuming that fungus flora characteristic of the 'goradu' soil might possess some selective antagonistic activity against pea rhizobia, fifty isolations of soil fungi from 'goradu' soil were made on peptone sucrose acid agar recommended by Waksman (1926), and were maintained on potato dextrose agar. All these isolates were then inoculated into 20 ml. of 2% maltose solution and Czapek's solution contained in 50 ml. Erlenmeyer flasks and allowed to grow for 25 days at 28° C. After that period the contents of the flasks were filtered through three layers of filter-papers to remove the spores and the filtrate tested for its selective anti-rhizobial activity by cup plate method on yeast extract mannitol agar,

Two cultures of pea Nos. 1 and 2, and two other cultures from 'shewri' (No. 5) and from 'guwar' (No. 7) were selected for this study.

It was found that only two isolates of fungi, both belonging to genus *Aspergillus* had some bacteriostatic effect on all the rhizobia tested. The growth of all the four isolates of rhizobia was inhibited up to a distance of 3 millimeters by filtrate of the cultures in maltose solution, and up to 6 millimeters by filtrates of the cultures in Czapek's solution. None of the other fungus isolates had any inhibitory effect on rhizobia and no selective antibiotic activity on pea rhizobia was also observed.

Effect of temperature on rhizobia.—Different species of rhizobia may behave differently towards temperature changes in the environment. The thermal death point of the various isotales in yeast extract mannitol medium was determined (presented in Table I) to know the effect of temperature on different species of nodule bacteria.

TABLE I

Thermal death points of the isolates in yeast extract mannitol medium

Isolate No.	Host plant	Lowest temperature at which no growth occurred
1	Pea (*Pisum sativum*)	56° C.
2	„ „	56° C.
3	„ „	56° C.
4	„ „	56° C.
5	'Shewri' (*Sesbania ægyptiaca*)	64° C.
6	Bean (*Dolichos lablab*)	62° C.
7	'Guwar' (*Cyamopsis tetragonoloba*)	66° C.
8	*Tephrosia purpurea*	60° C.
9	*Zornia diphylla*	58° C.
10	Lucerne (*Medicago sativa*)	64° C.
11	Berseem (*Trifolium alexandrinum*)	60° C.

Thermal death points of pea rhizobia in soil.—Since the organisms have their natural habitat in the soil, it was considered desirable to study their thermal death point also in soil. For this investigation 'goradu' soil where the pea fails to grow and the black soil where it grows were taken and the results are given in Table II.

TABLE II

Thermal death point of pea rhizobia in soil

Isolate No.	Host plant	Thermal death point in	
		Black soil	'Goradu' soil
1	Pea	60° C.	58° C.
2	,,	60° C.	58° C.
3	,,	60° C.	58° C.
4	,,	60° C.	58° C.

The above results clearly indicate that the pea rhizobia has a lower thermal death point (58° C.) in 'goradu' soils than in black soil, 60° C. Data on the mechanical analysis of the two soils obtained from the Chemistry Section of the Institute are presented in Table III.

The main differences are to be found in the sand and clay fractions. The lower sand content and the higher clay content in black soil as compared to 'goradu' soil, perhaps provide some protection from heat to rhizobia, whereas in 'goradu' soil the higher sand content and its non-sticky nature make the bacteria more susceptible to heat. Assuming that the sand content of the soil may be an important factor for the lower thermal death point in 'goradu' soil, the study of decrease in rhizobial population in the two types of soils along with sand at a particular temperature within a specific period was undertaken. Pea rhizobia inoculated into 'goradu' soil, black soil and sand were exposed to 54° C. (a temperature lower than their thermal death point) for 10 minutes and the number of viable cells surviving was determined by pour plate method (Table IV).

Table IV clearly shows that the number of viable cells after exposure at 54° C. for 10 minutes is least in sand and highest in black soil and the number in 'goradu' soil is in between the two. Repeated experiments have confirmed these results.

TABLE III

Mechanical analysis of 'goradu' and black soil (Percentage on air dry basis)

		'Goradu' soil	Black soil
1.	Moisture	1·53	6·68
2.	Organic matter	0·93	1·70
3.	Calcium carbonate	0·40	0·40
4.	Coarse sand	0·88	0·55
5.	Fine sand	80·52	23·56
6.	Silt	4·50	35·25
7.	Clay	9·93	37·25
8.	Total soluble salts	0·02071	0·02231
9.	pH	7·6	7·7
10.	Colour	Light brown	Black
11.	Stickness	Non-sticky	Sticky

TABLE IV

The number of viable cells of rhizobia after exposure at 54° C. for 10 minutes in sand, 'goradu' soil and black soil

No.	Host plant	No. of organisms initially added to each tube	Number of viable organisms after exposure at 54° C. for 10 minutes in		
			Sand	'Goradu' soil	Black soil
1	Pea ..	1,020,000	535	868	1,100
2	,, ..	1,340,000	288	590	650
3	,, ..	1,840,000	368	551	893
4	,, ..	1,650,000	187	455	550
5	'Guwar'	2,800,000	1,133	8,081	11,450

Discussion

The study of fifty soil fungi isolated from 'goradu' soil to see if they had any selective antibiotic activity against pea rhizobia, did not reveal any such activity, thereby indicating that there was no phenomenon of selective antibiosis. From the thermal death points of the isolates in yeast extract mannitol broth (Table I) it can be easily noted that pea nodule bacteria are the least tolerant to high temperature (thermal death point 56° C.) and the 'guwar' bacteria the most resistant to heat (thermal death point 66° C.). All the isolates other than pea rhizobia, indigenous to 'goradu' soil are more resistant to heat with their thermal death points higher than for pea rhizobia. Furthermore, it is interesting to note that the thermal death point of pea bacteria is higher in black soil (60° C.) than in 'goradu' soil (58° C.). This raises a question as to why such a difference exists between the two soils. The answer to this may be found in information provided in Tables II and IV. The number of pea rhizobia is reduced to a minimum in sand after exposure at 54° C. for 10 minutes whereas it is at a maximum in black soil with 'goradu' soil in between the two. Similar values were also obtained for 'guwar' rhizobia but the number of viable cells present in all the three substrates remain much higher than in the case of pea bacteria. The observations indicate that sand provides the least protection from heat to rhizobia, while the black soil affords the highest degree of protection from heat. The reasons for the higher protective ability against heat of clay are its higher moisture retaining capacity and its sticky nature, and sand lacks these characters. The higher the sand content of the soil, the lesser is its protective ability from heat for bacteria living in it. The black soil contains 72·9% of clay and silt fractions which is nearly five times that in 'goradu,' 14·43%, and its moisture content on air dry basis is also nearly five times that of 'goradu' soil. These considerations justify the conclusion that sand contents of 'goradu' soil may account for the non-availability of pea rhizobia therein.

Although the soils are different, the average atmospheric temperatures in the months of April, May and June are as high in Charotar as in Broach and Surat Districts. Temperatures varying from 42–46° C. are recorded from the beginning of April up to third week of June in those areas. Since pea rhizobia is however more sensitive to temperature changes it is probable that the number of bacteria get reduced at a greater rate in 'goradu' soil as compared to black soil. The number of pea rhizobia evidently gets so much reduced at the end of summer that they are not able to nodulate plants extensively. There is no reliable method available by which they

can be readily isolated from soil as such and population studies extended to cover different seasons of the year. From the present studies, it seems to be quite reasonable to infer that the cause of the failure of pea rhizobia to survive longer in 'goradu' soil lies in the nature of the soil, namely, its high sand content. That this does not occur in the case of other rhizobia may be that their number is not reduced to the same extent and they can multiply quickly under favourable conditions and form nodules in sufficient numbers on their respective host plants.

SUMMARY

The failure of pea (*Pisum sativum*) to grow in 'goradu' soil of Central Gujarat without artificial inoculation of the seed with the specific root nodule bacteria has been investigated. The study of a large number of soil fungi isolated from 'goradu' soils failed to show that they had any selective antibiotic activity against the pea rhizobia. It has been explained that the high sand content of this soil and the lower thermal death point of the pea rhizobia are the probable causes for the failure. The high sand content does not give any protection to the pea rhizobia against the high temperatures of the summer months with the result that their number gets so reduced and are not able to nodulate plants sufficiently.

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THE SOURCE OF ACETYLCHOLINE IN THE FROG'S HEART ON DIRECT ELECTRICAL STIMULATION

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Direct electrical stimulation releases acetylcholine from the stomach of dog and frog (Singh, Sharma and Bhatnagar, 1959 *b*) and from the frog's heart (Singh, Singh and Bhatnagar, 1960) after the vagal nerve endings have fatigued. These tissues contain ganglia, and it is possible that the acetylcholine might originate from the ganglion cells and not the muscle fibres. The possibility of origin of acetylcholine from the muscle fibres is suggested by the presence of cholinestrase in the muscle cells as shown by histochemical technique (Koelle, 1950, 1951; Holmes, 1957; Giacobini, 1959). In the present research an attempt has been made to find whether the acetylcholine originates from the ganglion cells or muscle fibres when the above tissues are directly stimulated with electric current. These experiments suggest that the acetylcholine originates from the muscle fibres.

Experimental

Frog's heart on direct electrical stimulation releases acetylcholine (Singh, Singh and Bhatnagar, 1960). The lower two-thirds of the ventricle do not contain ganglion cells (Lovatt Evans, 1956); this we have confirmed in these experiments. The heart of the frog, *Rana tigrina*, was taken out and two pieces were made by cutting the ventricle. One piece consisted of sinus venosus, auricles and the upper half of the ventricle, and the other piece, of the lower half of the ventricle. To get at least one gram of the lower half of the ventricle, tissues from four hearts had to be pooled together for each experiment.

The pieces from the hearts were washed free of blood in eserined Ringer's solution for half-an-hour. After this preliminary washing, they were aerated for one hour in 12 c.c. of fresh eserinised Ringer's solution to study the liberation of acetylcholine by the unstimulated pieces. They were then electrically stimulated with induction shocks for one hour in a muscle chamber

containing 12 c.c. of eserinised Ringer's solution, as described previously (Singh, Singh and Bhatnagar, 1960). These solutions were assayed for acetylcholine on leech muscle, sensitive to 1 in 1,000 million acetylcholine. In some experiments, the acetylcholine content of heart muscle was determined as described previously (Singh, Sharma and Bhatnagar, 1959 *b*).

RESULTS

The unstimulated heart pieces.—The spontaneously beating frog's heart does not release acetylcholine, so that the release of this substance is not necessary for rhythmicity (Singh, Singh and Bhatnagar, 1960). In contrast, cut-pieces of both auricles and ventricles may release acetylcholine (Table I). The release of acetylcholine is therefore clearly due to injury caused by cutting. This shows how necessary it is to avoid injury to tissues in studying release of acetylcholine.

Effect of stimulation.—Electrical stimulation of lower half of the ventricle causes greater release of acetylcholine per g./wt. than similar stimulation of the piece consisting of the sinus venosus, auricles and the upper half of the ventricle (Table I). This was rather surprising, so it was decided to estimate the acetylcholine content of these pieces. It was found that the upper piece consisting of the sinus venosus, auricles and the upper half of the ventricle contained less acetylcholine per g./wt. than the lower half of the ventricle (Table II). This clearly proves that the heart muscle fibres contain acetylcholine, rather more than nervous tissue.

DISCUSSION

The ganglion-free portion of the ventricle releases more acetylcholine per g./wt. than the rest of the heart which contains ganglion cells. It is clear therefore that acetylcholine chiefly originates from the muscle tissues and not the nervous tissue in the heart.

Then what is the function of acetylcholine in the heart? The frog's heart does not release any acetylcholine while beating spontaneously (Singh, Singh and Bhatnagar, 1960). Extracellular acetylcholine is therefore not necessary for rhythmicity. Rabbit's auricle releases acetylcholine while beating rhythmically (Burn, 1956); this is probably an overflow. This is similar to the phenomenon shown by frog's stomach muscle and dog's stomach muscle; the latter always releases acetylcholine while at rest, but the former may not (Singh, Singh and Bhatnagar, 1959 *a*, *b*). It appears that mammalian tissues are rather more delicate than frog's tissues and are thus more liable to release acetylcholine than frog's tissues.

TABLE I

Acetylcholine released from two pieces of frog's heart during electrical stimulation. One piece consisted of lower half of the ventricle and the other, the remaining portion of the heart consisting of sinus venosus, auricles and upper part of the ventricle

No. of Experiment	Acetylcholine in nanograms released from 1 gm. of tissue per hour			
	From sinus venosus, auricles and upper half of ventricle		From lower half of ventricle	
	During resting condition	During stimulation	During resting condition	During stimulation
1	14	320	17	876
2	18	1064	27	1627
3	0	710	0	790
4	110	660	300	1505
5	140	563	0	1216
6	215	646	154	1081
7	300	600	467	93
8	0	180	90	360
9	56	840	10	1421
10	109	875	200	1202
11	0	900	0	1337
12	10	427	300	1000
13	30	140	40	1000
14	0	130	0	941
15	0	208	0	192
16	75	900	39	1186
17	100	900	70	1500
18	0	55	0	80
19	16	64	37	150
20	153	1541	440	3122
21	53	911	0	2051
22	0	888	0	1700
23	51	1770	25	2770
24	100	880	200	1700
Average ..	64	670	101	1240

Acetylcholine has therefore two possible functions in the heart; it is either released during stimulation of the vagus nerve to produce inhibition or it has some other intracellular function. It is quite possible that the acetylcholine released on stimulation of the vagus nerve comes not from the vagal nerve endings but from the muscle fibres. If the frog's heart is immersed in Ringer's solution containing various concentrations of acetylcholine up to 1 in 10,000, it may continue to beat and yet on stimulation of the vagus nerve, complete inhibition is produced (Singh, Sehra and Singh, 1945). The same

TABLE II

Acetylcholine content of two pieces of frog's heart. One piece consisted of lower half of the ventricle and the other of the remaining portion of the heart, consisting of sinus venosus, auricle and upper half of the ventricle

No. of Experiment	Acetylcholine content, μg./g. of tissue	
	Sinus venosus, auricle and upper half of ventricle	Lower half of ventricle
1	6	9
2	7·3	9·2
3	5·8	9·2
4	5·8	9
5	5·4	8
6	6·4	9·2
7	5·6	7·8
8	7·8	10
9	6·5	10·2
10	5·4	8·8
11	5·6	10·8
12	7	11·4
13	7	11
14	6·4	10·6
15	3·2	9
16	3·6	9·2
17	7·8	9
Mean ..	6	9·5

has been found on mammalian heart (Obrink and Essex, 1953); vagal stimulation could stop the heart even when it was beating under the influence of high concentrations of acetylcholine (up to 1 in 100). This can be interpreted in two ways: either the nerve did not liberate acetylcholine, or liberated it within an area into which the acetylcholine from outside had difficulty in penetrating. In the frog's heart this is unlikely, as the tissue is thin and transparent; at least the experiment excludes the extracellular release of acetylcholine by the vagal nerve endings.

The view that nerves may release chemical substances from the tissue cells is not unreasonable, as sympathetic nerves actually release noradrenaline and adrenaline from the adrenal medulla; the cells of the adrenal medulla may be related to nerve cells developmentally but they are not nerve cells. The discovery of many active substances in various organs also supports the above view. Thus, besides acetylcholine, adrenaline and noradrenaline, other substances have been discovered which are suspected to play a role in transmission at nerve endings and synapses; these are histamine, 5-hydroxytryptamine, gamma aminobutyric acid. The chemical theory of transmission by liberation of active substances at nerve endings was reasonable so long it was restricted to two substances, acetylcholine and noradrenaline; and two kinds of nerves were postulated, cholinergic and adrenergic. But with the discovery of more active substances in tissues, more kinds of nerves have to be postulated, such as histaminergic, etc. More active substances are likely to be discovered in tissues and so further classification of nerves will be required. It is likely therefore that there is only one kind of nerve liberating different substances from tissue cells which contain these substances.

In contractile cells, acetylcholine may have some role in contraction. This is suggested by the observation that factors, both physical and chemical, which increase the mechanical response of dog's stomach muscle, also increases its acetylcholine content (Singh, Sharma and Bhatnagar, 1960). Further, if frog's stomach muscle is heated to 60° C., it releases acetylcholine and also relaxes actively, suggesting that acetylcholine had kept the contractile mechanism from relaxing, and is thus, in some way, involved in contraction (Singh, Sharma and Bhatnagar, 1959 *a*). Acetylcholine probably neutralises active relaxation in frog's stomach muscle, and thus enhances the mechanical response, as in this muscle, contraction and active relaxation appear to occur simultaneously (Singh and Singh, 1956). The occurrence of simultaneous active relaxation with contraction will diminish the mechanical response, so that if it is antagonised, the mechanical response will increase. This may explain the poor acetylcholine content of striated muscle, as it

relaxes passively. As the heart muscle fibres contain acetylcholine, they probably relax actively.

SUMMARY

1. In frog's heart, on electrical stimulation, more acetylcholine per g./wt. is liberated from the lower half of the ventricle which is free from ganglion cells, than from the rest of the heart which contains ganglion cells. This suggests that acetylcholine mainly originates from the muscle cells.

2. The acetylcholine per g./wt. content of the ganglion free portion of the ventricle is greater than the rest of the heart which contains ganglia. This shows that acetylcholine is also contained in muscle cells besides nerve cells.

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THE SKULL OF *HEMIRHAMPHUS XANTHOPTERUS* CUV. & VAL.

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I. Introduction

The morphology of the skull of Indian Teleosts has not attracted attention of many workers. Sarabhai (1932) described the endoskeleton of the common edible fish *Labeo rohita*. In the same year, Bhimachar published a detailed account of the cranial osteology of *Ophicephalus striatus*, which actually is an improvement upon an old paper by Day (1914) on the osseous system of *Ophicephalus striatus*. In the description of the endoskeleton of *Otolithus*

ruber, Dharamrajan (1936) has included its skull as well. However, greater attention has been drawn to the cranial morphology of fishes by Ramaswami (1945, 1946, 1948, 1951 *a*, 1951 *b*, 1952 *a* 1952 *b*), who has dealt with the chondrocranium and osteocranium of *Gambusia*, *Oryzias*, *Aplocheilus*, *Xiphophorus*, *Homaloptera*, *Gyrinocheilus*, *Psilorhynchus* and *Balitora*. Srinivasachar (1953) worked out the development of chondrocranium of *Ophicephalus* and also described the skull of the same (1955).

As far as we have been able to ascertain, none of the skulls of the Indian Hemirhamphidæ has so far been described. Of a few references available, one is from Gregory (1933) who in his comprehensive dissertation on 'Fish Skull' has drawn attention to the close similarity between the Cyprinodont fishes and *Hemirhamphus* of the Synentognathi. The other available account is from Starks (1926) who has included the description of the ethmoid region of *Criodorus atherinoides* (Hemirhamphidæ) in his account of the bones of the ethmoid region of the fish skull.

II. Material and Method

Some forty specimens of *Hemirhamphus xanthopterus* Cuv. and Val. were collected by students of Government College, Ajmer, at Madras beach, in October, 1955. The specimens were kindly made available to us for investigation. The skulls were carefully cleaned by macertation and boiling in 5% solution of ammonium carbonate (Cantuel, 1949). A few Alizarin preparations were also made. The various structures were studied with the help of 'Bull Eye' lens and the diagrams were made free hand.

III. Observations

(*a*) *The Ethmoid Region.*—In *Hemirhamphus xanthopterus* the median ethmoid is clearly visible on the dorsal surface of the head and is firmly articulated with the frontals posteriorly. It is divided into a left and a right portion by an antero-posteriorly running ridge continuous with the median line dividing the skull dorsally into bilaterally symmetrical halves. The lateral ethmoids are in the form of small oval depressions on either side of the median ethmoid. The median ethmoid is purely a cartilaginous structure while the lateral ethmoids are osseous. There is no distinction between the anterior and posterior portions of median ethmoid in *Hemirhamphus xanthopterus*, nor is there any difference in the two regions as far as ossification is concerned. This offers a contrast to Day's (1914) description of the median ethmoid region of the skull of *Ophicephalus striatus*. According to his description the 'supraethmoid' is a light spongy bone and the ethmoid articulates with the nasals dorsally. But the supraethmoid and ethmoid as present in

Ophicephalus striatus are not distinguished in *Hemirhamphus xanthopterus*. Bhimachar (1932), however, described Day's (1914) supraethmoid and ethmoid as only supraethmoid and mentioned it as a poorly ossified bone.

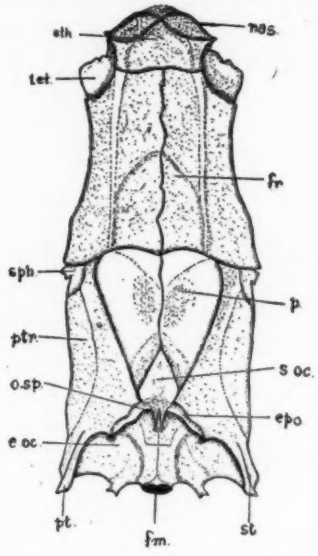

FIG. 1

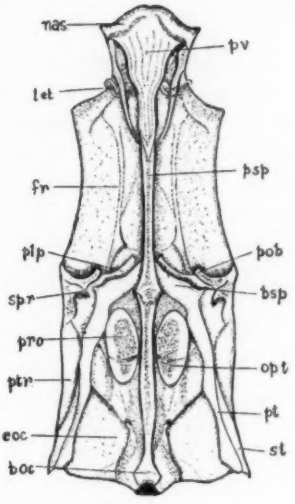

FIG. 2

FIG. 1. Dorsal view of the skull.
FIG. 2. Ventral view of the skull.

This statement is not correct in the case of *Hemirhamphus xanthopterus* as the ethmoid bone is purely cartilaginous. Kindred (1919) and de Beer (1937) called the median ossified bone in the nasal region as ethmoid and the intramembranous paired bones as supraethmoids. This description too, will not hold good in the case of *Hemirhamphus xanthopterus*, as there are no intramembranous paired bones in the ethmoid region of this fish.

The lateral ethmoids are observed as oval bony structures on either side of the ethmoid region. They can as well be called prefrontals or ectoethmoid bones; names usually used by Day (1914) and Bhimachar (1932).

Ventral to the ethmoid and anterior to the parasphenoid in the ethmoid region, lies the median prevomer. The prevomer is essentially a component of the palate. It does not bear prevomerine teeth. The presence of a single median prevomer in *Hemirhamphus xanthopterus* is an interesting feature. Goodrich (1930) described a single prevomer in *Acipenser* and some Teleosts

and is of the opinion that the median prevomer is a derivative of paired fused prevomers. However, Gaupp (1905) has stated that the median prevomer shows signs of paired origin in *Salmo* while Walther (1883) has shown that in *Esox* it arises from paired rudiments. In *Hemirhamphus xanthopterus*, the prevomer directly articulates with the parasphenoid posteriorly and gives out a projection as in *Gyrenocheilus* (Ramaswami, 1952).

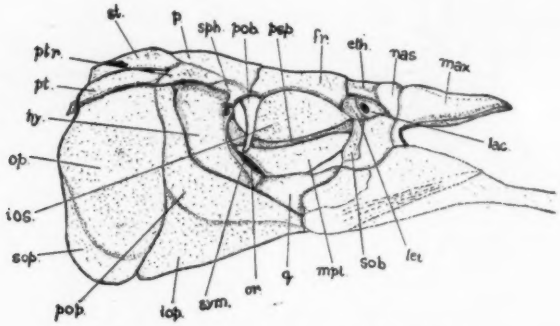

FIG. 3. Lateral view of the skull.

(*b*) *The Orbito-temporal Region.*—Posterior to the ethmoid region on the dorsal side of the skull is the frontal region consisting of paired frontals. The frontals are elongate, transparent and cartilaginous structures forming nearly one-third of the total length of the skull. They are not separated from each other posteriorly by the intervention of the supraoccipital as in *Ophicephalus striatus* (Srinivasachar, 1955). The posterior portion of the frontals is broader than the anterior and articulates with the anterior end of the parietals.

The cartilaginous parietals are paired and articulate anteriorly with the frontals. The pterotic bones of the auditory region fuse with the parietals. The anterior end of the supraoccipital penetrates into the mid-parietal region and separates the two parietals in the posterior region.

The orbits are roofed over by the paired frontals. They are separated from each other by a membranous interorbital septum which is connected ventrally with the parasphenoid and posteriorly with the pleurosphenoids. The orbit is not bounded ventrally by sub-orbitals. There is single sub-orbital in *Hemirhamphus xanthopterus* which, in view of its location, be better referred to as post-orbital. The ventral boundary of the orbit is supported by the osseous pterygoid which fuses with the quadrate ventrally and parasphenoid dorsally just below the interorbital septum. Posterior to the

pterygoid is another characteristic bone, the symplectic which constitutes the ventral boundary of the orbit.

The lacrimal is a distinct osseous element at the antero-latero-dorsal margin of the orbit. A distinct lacrimal foramen is also noticed. Ramaswami (1952) named the anterior sub-orbital as lacrimal in *Gyrinocheilus*, *Garra*, and *Crossocheilus*.

The sphenoid region, consisting of paired basisphenoids, a parasphenoid and paired lateral pleurosphenoids, lies immediately posterior to the orbital region. The basisphenoid is a small bone and lies between the two enlargements of the pro-otic bones. The parasphenoid is truncate and arches over the ventral side of the interorbital septum. Anteriorly the parasphenoid fuses with the prevomer. The pleurosphenoids are the laterally disposed cartilaginous extensions of the basisphenoid and with the help of the latter form the hypophysial fenestra. The pleurosphenoids have been referred to

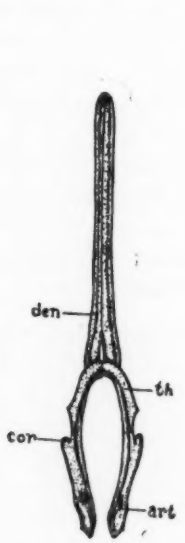

FIG. 4

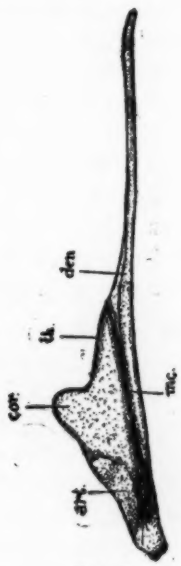

FIG. 5

FIG. 4. Dorsal view of the lower jaw.
FIG. 5. Lateral view of the lower jaw.

as the alisphenoids by Day (1914) and Bhimachar (1932). Goodrich (1930), however, is of the opinion that this bone in fishes is neither homologous with the mammalian alisphenoid, since it is an ossification in the primitive cranial wall of the cavum epitericum, nor can it be the exact homologue of the pleuro-

sphenoid of reptiles and birds. The true nature of the pleurosphenoid has been described by Goodrich (pp. 436–37, 1930).

(*c*) *The Auditory Region.*—The auditory region comprises paired opisthotics, pro-otics, sphenotics, supratemporals, post-temporals, and pterotic bones. The pro-otics form the anteriormost region of the auditory capsule and lie immediately posterior to the basisphenoid. Each pro-otic is oval in outline and has the appearance of tympanic bulla of mammals. The pro-otics are completely ossified and are the most osseous of all the bony elements of the skull. Posterior to pro-otics lie the opisthotics, one on either side and fused with the pro-otics. The fused pro-otics and opisthotics form the auditory capsule. The sphenotic is, however, separated and does not take part in the formation of the auditory capsule. Srinivasachar (1955) mentions that the opisthotic does not associate itself with the sphenotic, pterotic and epiotic in the formation of the auditory capsule in *Ophicephalus striatus*. In *Exocœtus* the pterotic and sphenotic ossify in the cartilaginous lateral wall of the auditory capsule and have intramembranous extensions, (de Beer, 1937). The sphenotic in *Hemirhamphus xanthopterus* is arch-shaped and lies ventral to the suborbital process of the frontal of the same side. From the lateral side of each sphenotic extends ventrally a process, the sphenotic process. The sphenotic process has so far not been noticed in any other Teleost. The pterotics are elongated antero-posteriorly and border the parietals on the inner side. Posteriorly the pterotics articulate with the post-temporals and the supratemporals, while anteriorly they articulate with the sphenotics. The epiotics are situated posteriorly near the supraoccipital spine and articulate anteriorly with the parietals.

(*d*) *The Occipital Region.*—The supraoccipital does not take part in the formation of foramen magnum. The posterior end of the supraoccipital bears a pair of supraoccipital spines. Ramaswami (1952) in *Garra mullay* and Srinivasachar (1955) in *Ophicephalus striatus* describe only a single median supraoccipital spine. As has already been mentioned, the supraoccipital penetrates into the posterior portion of the parietals. The exoccipitals extend dorsally to cover the foramen magnum. The occipital condyle is a bony enlargement and situated at the postero-ventral end of the foramen magnum. The basioccipital is a small bony extension of the parasphenoid.

(*e*) *The Opercular Region.*—The opercular region consists of paired operculars, subopercular, interopercular and preopercular. The opercular is oval in outline and forms the postero-lateral part of the head. The anterto-dorsal extremity of the opercular is in contact with the pterotic of the corresponding side. The ventral and ventro-lateral borders of the oper-

cular form dense cartilaginous portions which have been recognised as suboperculars in *Hemirhamphus xanthopterus*. The interopercular is triangular and lies between the opercular and preopercular. It forms the postero-ventral boundary of the skull. The preopercular which is also triangular in outline extends dorsally up to the pterotic of the temporal region while anteriorly it articulates with dentary below the quadrate.

(*f*) *The Upper Jaw*.—The upper jaw composed of fused premaxillaries is a triangular structure. The base of the triangle lies parallel to the median ethmoid. The premaxillary bears small conical projections which are not arranged in definite linear rows. These maxillary denticles are more numerous on the anterior side than the posterior. Two ramii of the maxillary bone are united by a continuous extension of the cartilage. At the base of the premaxillary triangle, distal to the ethmoid bone, are present paired nasals which extend outwards laterally and fuse with the lateral ethmoids.

(*g*) *The Lower Jaw*.—In the lower jaw of *Hemirhamphus xanthopterus* only two elements, the articular and the dentary, are distinct. Of these two the dentary is very conspicuous. It is much elongate and forms with its counterpart, a long beak-like structure. There is no mento-meckelian ossification on the dentary. The posterior parts of the two ramii, which border the mouth, bear conical denticles. The articular is relatively small and elongated. It articulates with the quadrate posteriorly which gives a short process anteriorly for this articulation. The posterior portion of the quadrate lies in contact with the anterior end of the preopercular and the hyomandibular. The hyomandibular is cartilaginous and articulates dorsally with the lateral surface of the auditory region, the major portion of the articulation being shared by sphenotic and the pterotic. The posterior edge of the hyomandibular cartilage articulates with the anterior edge of the preopercular. The hyomandibular, being in articulation with the quadrate anteriorly, supports the lower jaw.

IV. Summary

The authors give a detailed description of the skull of the fish *Hemirhamphus xanthopterus* Cuv. and Val. The more important points described are as follows:—

1. The median ethmoid and the lateral ethmoids are alone present, the supraethmoid is not differentiated.

2. The prevomer is edentulous and gives out a posterior projection which articulates with the parasphenoid.

3. The elongated and paired cartilaginous frontals are not separated from each other.

4. The parietals are partly separated from each other by the intervention of the supraoccipital.

5. The interorbital septum is membranous.

6. The suborbitals are absent.

7. The ventral boundary of the orbit is formed by an osseous pterygoid and symplectic.

8. The sphenoid region comprises paired basisphenoids, a median parasphenoid and paired lateral pleurosphenoids.

9. The small supraoccipital bears posteriorly a pair of supraoccipital spines.

10. The opercular region consists of paired operculars, subopercular, interopercular and preopercular.

11. The upper jaw is formed only of the premaxillaries which bear small, irregularly disposed conical teeth.

12. The lower jaw is prolonged to form a long slender half-beak. The angular is absent. The Meckel's cartilage is not ossified.

V. ACKNOWLEDGMENTS

We express our gratitude to Shri Bhim Sen, Principal, Government College, Ajmer, for research facilities. Our thanks are also due to Shri Bhanwar Lal Garg for assistance in the preparation of the illustrations.

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VII. Abbreviations

art., articular
boc., basioccipital
bsp., bassiphenoid
cor., coronary process
den., dentary
eoc., exoccipital
epo., epiotic
eth., ethmoid
fm., foramen magnum
fr., frontal
hy., hyomandibular
iop., interopercular
ios., interorbital septum
lac., lacrimal
let., lateral ethmoid
max., premaxillary
mc., Meckel's cartilage
mpt., pterygoid
nas., nasal
op., opercular
or., orbit
osp., occipital spine
p., parietal
plp., pleurosphenoid
pob., postorbital
pop., preopercular
pro., pro-otic
psp., parasphenoid
pt., post-temporal
ptr., pterotic
pv., prevomer
q., quadrate
sob., suborbital
soc., supraoccipital
sop., subopercular
sph., sphenotic
spr., sphenotic process
st., supratemporal
sym., symplectic
th., teeth

STUDIES ON THE MICROBIAL SPOILAGE OF CANNED FOOD

III. Isolation of Thermophilic Cocci From an Imported Canned Fish

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INTRODUCTION

IN the earlier studies on the microbial spoilage of canned food the authors reported on the isolation of five species of *Bacillus*, one of *Clostridium* and one of *Lactobacillus*, from canned fruit and vegetable products (Rangaswami and Venkatesan, 1959). In the present report the details on the spoilage of imported canned fish due to a coccus type of bacterium is presented.

MATERIAL AND METHODS

During August 1959 a fish can showing signs of swelling was collected from a grocery in Chidambaram, Madras State. This can was packed with the fish, 'Herrings', in tomato juice. As noted on the can it was packed in the month of February, 1959.

The can was thoroughly examined, following the techniques detailed earlier by the authors (Rangaswami and Venkatesan, 1959). The microbial population in the canned fish was estimated by the dilution plate technique. For testing the spoilage capacity of the bacterial isolate, freshly caught 'Chel Kendai' variety of fish (*Labeo fimbriatus* Altson) was packed in A $2\frac{1}{2}$ cans and sterilized in pressure cooker at 15 lb. pressure for 15 minutes and sealed in a double seamer; a pure culture of the test organism was inoculated into the can just before sealing. On sealing, the cans were incubated at 28° and 38° C. for 45 days and were examined subsequently by conducting a regular cut-out test (Anonymous, 1956).

The organism was studied for morphological, cultural and physiological properties, in the usual manner and its systematic position determined with the help of Bergey's *Manual of Determinative Bacteriology* (Breed *et al.*, 1957). The heat-resistant quality of the bacterium was studied both in the liquid and agar cultures, adopting the procedure followed by the authors in their earlier work (*loc. cit.*).

In order to find out the optimum pH range for growth of the organism, nutrient agar, adjusted to different pH levels, was prepared in the usual manner, poured in petri-dishes and the test organism inoculated by streaking all over the surface of the medium. The dishes were incubated for four days at room temperature and the intensity of growth of the organism recorded.

To find out the thermal death point of the bacterium, a ten-day old broth culture was diluted in sterile distilled water to get a uniform suspension. This bacterial suspension was distributed in 10 ml. quantities in sterile test-tubes. The tubes were kept in a water-bath and were heated to different temperature levels for one minute. Subsequently the tubes were cooled by immersing in cold water, and the contents plated in nutrient agar medium by the dilution plate technique. The number of colonies developing in the medium in each case was recorded and the thermal death point obtained.

Experimental Results

1. *Nature of spoilage.*—Externally, the can exhibited swelling of springer type, but there was no irregular malformations in its shape or apparent leakage, slit or openings in any portion. The contents of the can did not show any putrefactive symptoms, nor the fish meat was digested to any considerable extent. Though there was no foul smell, the product seemed to have lost the fishy odour and minute air-bubbles were observed in the pasty portions, immediatley after opening. The metal of the can was highly corroded. The product was acidic, of pH 4·7. When representative samples of the contents, both from the pasty and solid portions, were plated in yeast extract dextrose, potato dextrose and nutrient agar media, bacterial colonies readily developed within 48 hours. Only one type of colony was observed in all cases and the bacterial population was estimated at 862,500/ml. of the product.

2. *Inoculation tests.*—In order to test the spoilage capacity of the bacterium, a pure culture of the bacterial isolate obtained above was tested on freshly cooked, sterilized and canned fish, following the procedure described under Material and Methods. After an interval of 45 days the inoculated cans were examined by conducting cut-out tests. The results are presented in Table I.

The bacterium was re-isolated from the inoculated and spoiled cans and in each case was found to be identical with the inoculum.

3. *Morphological, cultural and physiological properties of the bacterial isolate.*—On nutrient agar, the bacterial colony was scanty, non-spreading, discrete and dirty yellow in colour. In nutrient broth, the growth was dull yellow coloured, slightly turbid, but no pellicle or sedimentation formed

even after 15 days. Growth on potato plug was slimy, dirty yellow, smooth and mucoid.

TABLE I

Results of cut-out tests of the inoculated fish cans

Characters examined	Inoculated with test organism			Check
	Can No. 1	Can No. 2	Can No. 3	
External appearance of the can	Swell	Springer	Springer	Normal
Internal appearance of the can	Corroded	Corroded	Corroded	Bright
Appearance of the fish product	Pasty	Pasty	Pasty	Solid
pH of the product	5·2	5·6	5·5	6·2
Bacterial population: millions/ml.	9·4	2·7	3·4	0
Smell	.. Fishy odour	Fishy odour	Fishy odour	Fishy odour

The bacterial cell was hyaline, minute, spherical, occurring in single or in short chains, 0·6 to 0·9 μ in diameter; capsulated, non-motile and without endospores. The bacterium was Gram-positive but not acid fast. When grown in liquid cultures, it did not reduce nitrate and failed to hydrolyse starch, or liquefy gelatin, but readily coagulated milk, producing acid in the medium in 48 hours, without the reduction of litmus in the milk. It did not produce ammonia or H_2S when grown in liquid broth. It gave positive indole, methyl red, Voges Proskauer and lipolytic activity tests. The organism was capable of utilizing arabinose, glucose, sucrose and lactose as carbon sources, with the production of acid in the media, but did not utilize matlose, raffinose, starch, glycerol, mannitol and sorbitol. The bacterium was microaerophilic in its oxygen relationship.

In a study on growth of the bacterial isolate in liquid and agar cultures at various incubation temperatures, it was found that there was optimum growth of the bacterium at 42° C., slight non-spreading growth at 48° and 38° C., very slow growth at 28° C. and no growth at 55° and 20° C.

On the basis of the above detailed characters, the bacterium is identified as *Streptococcus thermophilus* Orla-Jensen, belonging to the family Lactobacillaceæ Winslow *et al.* (Breed *et al.*, 1957).

4. *Optimum pH for the growth.*—The bacterium was tested for its growth at pH levels ranging from 3·5 to 8·5. It was found to grow between pH 3·5 to 7·0, and optimum growth was observed at pH 5·0 to 6·0. There was no growth above pH 7·5.

5. *Thermal death point.*—The bacterium was tested for heat resistance, by heating to various temperatures ranging from 100 to 212° F. as detailed under Material and Methods and the results are given in Fig. 1.

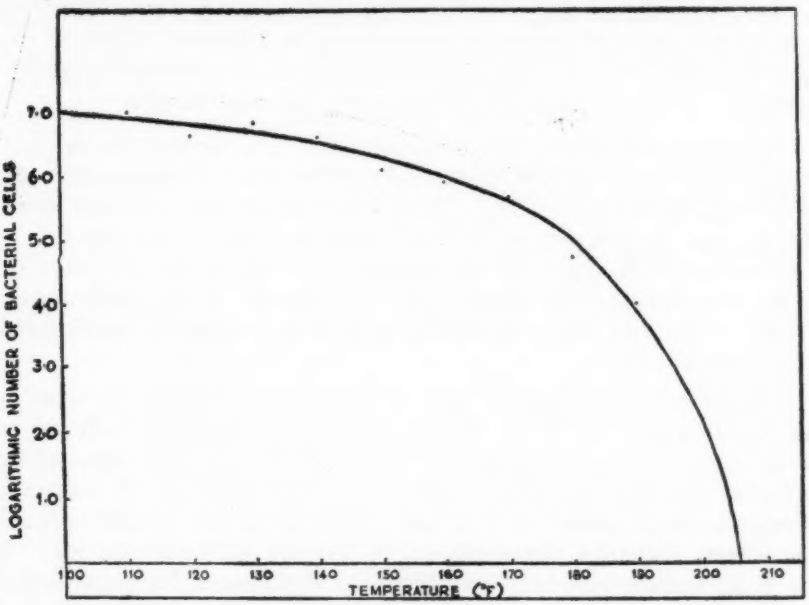

FIG. 1. Thermal death point and survivor curve for *Streptococcus thermophilus.*

It was found that the bacterium was not much affected by heating up to a temperature of 180° F. but was completely destroyed by heating for one minute at 210° F., indicating thereby the fair resistance put forth by the bacterium at medium temperatures.

DISCUSSION

The spoilage quality of *S. thermophilus* on the canned fish is clearly indicated in the present studies. Though other species of *Streptococcus* are

reported to cause damage to canned food, the present report seems to be the first record of *S. thermophilus* causing spoilage of canned food. Of the many types of spoilage bacteria in canned food, cocci are not so very common. Also, they do not form endospores, and so are relatively less resistant to heat and are easily killed in the routine food processing. According to Jones (1949) the spoilage caused by non-sporing cocci is of common occurrence in the cans packed with animal and marine products. Tanner (1944) isolated many of the non-heat resistant bacteria such as cocci which were found in the cans processed under conditions which would normally be expected to destroy such types. In India some of the spoiled commercially canned fish were examined by Venkataraman and Sreenivasan (1955) and were found to be spoiled by the aerobic as well as anaerobic bacteria, but no cocci were isolated by them. According to Baumgartner and Hersom (1956) the cocci may be entrapped in the fatty media and may survive even at temperatures sufficient to destroy resistant spore-forming spoilage bacteria.

In the present investigation the organism, *viz.*, *S. thermophilus* was found to resist heat up to 205° F. and also to grow better in an acid medium than in neutral or alkaline medium. It is probable that the organism entered the can and survived due to defective canning process. Also, the fish was preserved in tomato juice, an acid medium. The pH of the juice when examined after spoilage was found to be 4·7. The pH of the tomato juice is usually acidic, and hence the medium was quite suitable for multiplication of the bacterium.

The coccus was found to grow at temperatures between 28 to 48° C., but failed to grow at 20° C. Perhaps it could not multiply inside the can in the country where it was packed, *viz.*, Scotland, but when imported into India could multiply due to favourable temperature conditions, resulting in the damage to the foodstuff. It is evident therefore that what might appear to be an inert bacterium under one set of temperature conditions, may turn out to be spoilage type under another set of conditions. The growth of the bacterium would have been perhaps arrested if it were packed in a neutral medium instead of in acidic tomato juice and stored at low temperatures after importing into India.

SUMMARY

A spoiled canned fish, imported from Scotland, was examined and the spoilage was found to have been caused by a cocci type of bacterium. The bacterium was brought into pure culture, studied for its morphological, cultural and physiological properties and on the basis identified as *Streptococcus thermophilus* Orla-Jensen. Its ability to cause spoilage was proved

by inoculating it into freshly processed fish cans. The strain was resistant to heat up to 205° F. and it failed to grow at 20° C. On its temperature relationships, explanation has been offered for its spoilage under Indian conditions and its inactivity under the conditions obtaining in the country where it was packed.

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STUDIES IN ERYTHROXYLACEÆ—I

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Introduction

The family Erythroxylaceæ comprises three genera, *viz.*, *Erythroxylum* (200 species), *Nectaropetalum* (4 species) and the monotypic *Aneulopha* (Lawrence, 1951). Bentham and Hooker (1862–1893) included *Erythroxylum* and *Aneulopha* in the family Linaceæ. However, Hutchinson (1926) and Engler and Prantl (1931) place them in a distinct family, the Erythroxylaceæ.

The floral anatomy of only *Erythroxylum coca* has been studied by Saunders (1937). Schnarf (1931) summarised the embryological work in the family. Later, Mauritzon (1934) studied the embryology of *Erythroxylum coca.*

The present paper deals with the floral anatomy of *Erythroxylum coca* Lam., *E. mooni* Hochr., and *E. monogynum* Roxb. and the embryology of the two latter species.

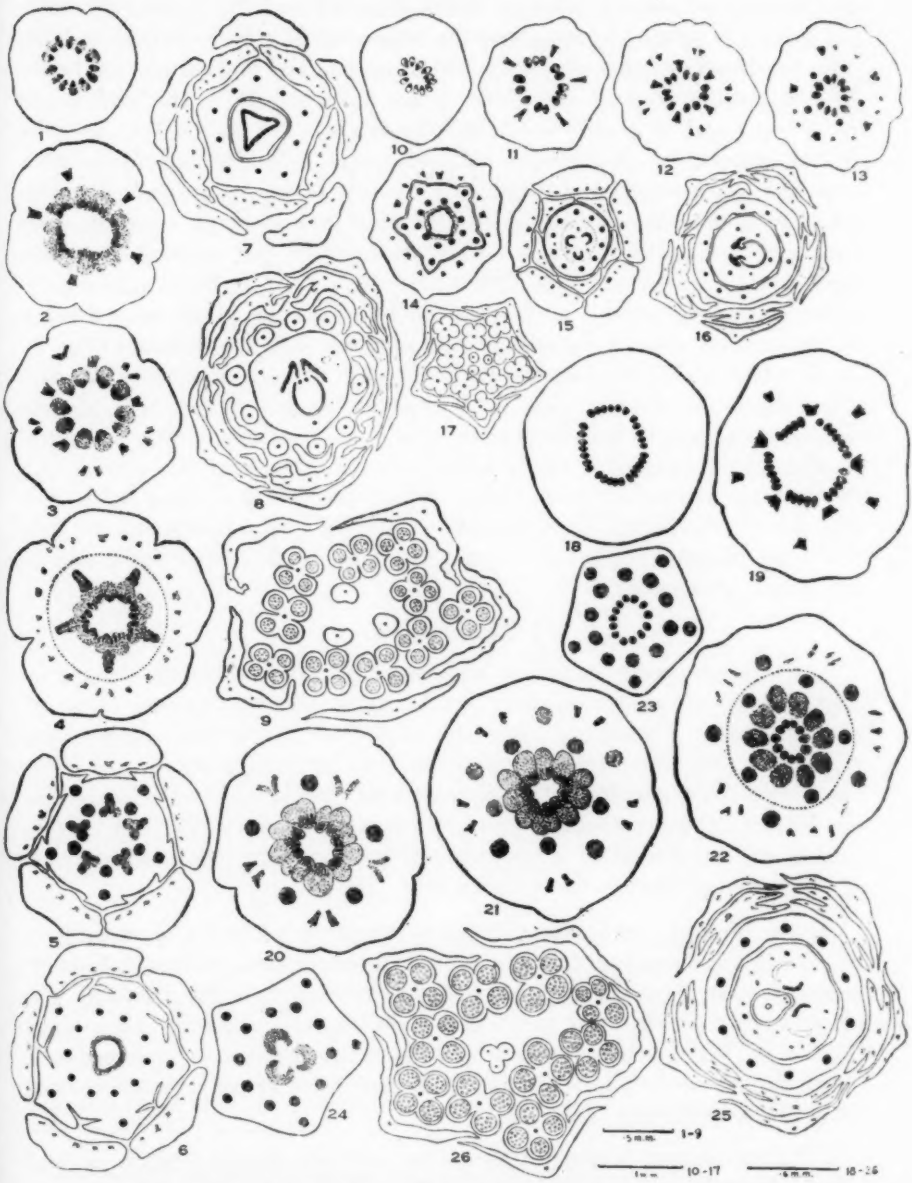
Materials and Methods

The materials were fixed in F.A.A. Usual methods of dehydration, infiltration and embedding were followed. The sections were cut at a thickness of 6–12 μ. Crystal violet and erythrosin combination was used for staining the sections.

Observations

Floral Anatomy.—The flower in the three species studied is pentamerous and bisexual. The petals on their inner sides bear ligule-like appendages (Figs. 8, 16 and 25). The stamens are united below into a tube and become free above (Figs. 7-9, 15–17, 25 and 26). Of the three carpels the fertile one bears a single ovule (Figs. 8, 16 and 25). The three styles are basally connate.

The pedicel shows a ring of vascular bundles (Figs. 1, 10 and 18). The conjoint sepal laterals and sepal midribs arise in two alternating whorls (Figs. 2, 3, 11, 12, 19 and 20). The former bifurcate radially and demarcate



FIGS. 1–26. Transections of flower passing from base upwards. Figs. 1–9. *Erythroxylum coca*. Figs. 10–16. *E. mooni*. Figs. 17–26. *E. monogynum*.

the marginal bundles of adjacent sepals (Figs. 12 and 20). Next, the traces for the petals arise alternating with the sepal midribs (Figs. 4, 13, 21 and 22). The bundles supplying the petals divide tangentially and supply the ligule-like outgrowths on their inner sides (Figs. 8, 16 and 25). The androecium in *E. coca* and *E. mooni* is obdiplostemonous. The staminal traces arise in two whorls, the antepetalous staminal traces becoming organised earlier than the antesepalous one (Figs. 4, 5 and 14). The antepetalous staminal traces in *E. coca* show adnation with the petal midribs (Figs. 4 and 5), while they are separate in *E. mooni*. In *E. monogynum* the staminal traces are demarcated in one ring (Figs. 22 and 23). In *E. coca* the floral stele is ring-like after the organisation of the staminal traces (Fig. 6). It becomes triangular at the level where the staminal tube separates from the thalamus (Fig. 7). In *E. mooni* and *E. monogynum* the main stele becomes split into three arcs from which the carpellary supply is organised (Figs. 15 and 24). Median dorsals are organised in *E. mooni* (Fig. 15). In all the three species the ventral bundles of the two sterile and fertile carpels unite and form two plexes. An ovular trace arises from each of these plexes so that the ovule is fed by two vascular bundles (Figs. 8, 16 and 25). Only the dorsal bundles extend into the stylar branches (Figs. 9, 17 and 26). The loculi of the sterile carpels are poorly developed.

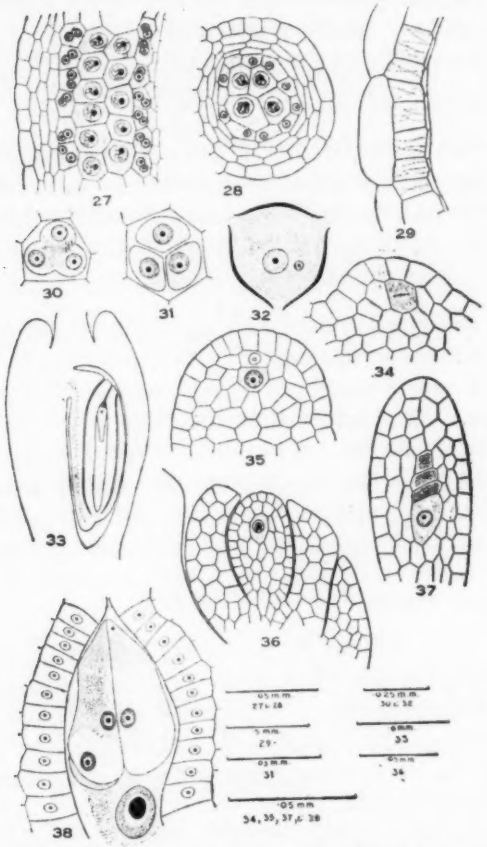
EMBRYOLOGY

Microsporogenesis and Male Gametophyte.—The wall of the developing anther shows an epidermis, three middle layers and the tapetum (Figs. 27 and 28). The hypodermal layer develops into the fibrous endothecium (Fig. 29). The cells of the secretory tapetum become binucleate and show vacuolated cytoplasm (Fig. 27); they become absorbed as the pollen grains are formed. Cytokinesis takes place by cleavage furrowing (Fig. 30). Pollen tetrads are tetrahedral in arrangement (Figs. 31). The tricolporate pollen at the shedding stage is two-nucleate (Fig. 32).

Ovule, Megasporogenesis and Female Gametophyte.—The ovule primordium which arises as a small protuberance grows and bends upwards during development. By the time the megaspore mother cell becomes differentiated, the integuments become well developed. The mature ovule is anatropous (Fig. 33). The nucellus is elongated and narrow and the inner integument forms the micropyle (Fig. 33). The innermost layer of the inner integument forms the endothelium (Fig. 38).

The hypodermal archesporium divides and cuts off the primary parietal cell and functions as the megaspore mother cell (Figs. 34–36). The primary parietal cell by further periclinal and anticlinal divisions forms a 2–3-layered

perietal tissue (Fig. 37). The megaspore mother cell (Fig. 36) gives rise to a linear tetrad (Fig. 37) of which the chalazal megaspore functions and



FIGS. 27–38. Figs. 28–30, 36 and 37. *E. mooni*. Figs. 27, 31–35 and 38. *E. monogynum*. Fig. 27. L.S. anther lobe showing wall-layers, tapetum and microspore mother cells. Fig. 28. T.S. anther lobe showing wall-layers, tapetum and microspore mother cells. Fig. 29. Fibrous endothecium. Fig. 30. P.M.C. showing cytokinesis. Fig. 31. Pollen tetrad. Fig. 32. Pollen grain at shedding stage. Fig. 33. Mature ovule. Fig. 34. L.S. ovule showing primary archesporium and its division. Fig. 35. L.S. ovule showing primary parietal cell and megaspore mother cell. Fig. 36. L.S. ovule showing megaspore mother cell. Fig. 37. Linear megaspore tetrad. Fig. 38. Micropylar part of the embryo-sac showing egg apparatus and polar nuclei; note endothelium.

develops into the embryo-sac according to the Polygonum type. The mature embryo-sac shows an egg apparatus consisting of two synergids which exhibit filiform apparatus and an egg (Fig. 38). The polar nuclei fuse before

fertilisation and the antipodals degenerate early. As the embryo-sac enlarges it completely crushes the nucellus and is surrounded by the endothecium.

DISCUSSION

A study of floral anatomy and embryology of Erythroxylaceæ reveals certain resemblances and differences with the Linaceæ.

The flower in both the families is fundamentally pentamerous with reduction in the number of carpels to three in Erythroxylaceæ. A similar tendency is seen in members of Linaceæ like *Radiola millegrana* and *Reinwardtia trigyna* (Saunders, 1937); in the former the flower is tetramerous throughout while in the latter the pistil shows three fertile carpels.

The petals in Erythroxylaceæ bear ligule-like appendages on their inner sides which are not present in Linaceæ. Obdiplostemony is seen in *Erythroxylum coca* and *E. mooni* while in *E. monogynum* the staminal traces are organised in one whorl. In Linaceæ there are three to five two-ovuled carpels. In Erythroxylaceæ, however, reduction is noticed in carpels and ovules. There is only one fertile carpel, the other two being represented by vestigial loculi. The ventrals of the fertile carpel unite with those of the sterile forming two plexes. From each, an ovular trace enters the funicle of the ovule in the fertile carpel. This surviving ovule appears to receive the vascular supply of the suppressed ovules.

According to Schnarf (1931) the anther tapetum in these two families is of the periplasmodial type. Mauritzon (1934), who studied *Radiola linoides*, described a secretory tapetum. The present study shows that the anther tapetum in *E. mooni* and *E. monogynum* is of the secretory type. The tricolporate pollen grains of Erythroxylaceæ differ from Linaceæ in the absence of ornamentations (Erdtman, 1952).

The ovules are anatropous, crassinucellate, and bitegmic in both Erythroxylaceæ and Linaceæ. Embryo-sac development in these families follows the Polygonum type; there is an endothelium. The antipodals in both the families degenerate early. However, a placental obturator which occurs in all the investigated species of Linaceæ is absent in Erythroxylaceæ.

Although the Erythroxylaceæ resembles Linaceæ in certain features of floral anatomy and embryology the former differs from the latter in having appendaged petals, a tricarpellary ovary with one fertile carpel, a double-traced ovule and absence of placental aobturator. These differences seem to justify their separation.

Heimsch (1942) who made a comparative study of wood anatomy of the families included in Gruinales and Terebinthales of Wettstein observes: "The Erythroxylaceæ should not be united with Linaceæ because of the general lack of the scalariform basal perforations, but the family is most closely allied to Linaceæ and Humiriaceæ on the basis of occurrence of tracheids and other xylem characters."

Summary

The floral anatomy of *Erythroxylum coca*, *E. mooni* and *E. monogynum* and the embryology of the two latter species have been studied.

The sepals are three-traced and the sepal laterals arise conjointly. The petals are appendaged and single-traced. Obdiplostemony is present in *Erythroxylum coca* and *E. mooni*. The ovule in the fertile loculus receives a pair of traces from the two plexes formed by the ventrals of the fertile and sterile carpels.

The tapetum is of the secretory type. The tricolporate pollen grains are two-nucleate at the shedding stage.

The ovules are crassinucellate, bitegmic and anatropous. An endothelium differentiated from the innermost layer of the inner integument surrounds the embryo-sac which develops according to the Polygonum type.

Acknowledgement

My thanks are due to Prof. M. Sayeeduddin for encouragement and guidance and Dr. C. V. Rao for kind help and criticism. My grateful thanks are due to Dr. K. Subramanyam, Regional Botanist, Southern Circle, for kindly going through the manuscript.

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A NEW SPECIES OF *XANTHOENCYRTUS* FROM INDIA

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Received March 26, 1960

Recently Nararayanan, Subba Rao and Kaur (1957) recorded a number of interesting genera belonging to the family Encyrtidæ parasitizing the pink mealy bug *Saccharicoccus sacchari* (Cockrell) from Delhi. Further study of the insects in question revealed that while *Astymachus* sp. could not be separated from *Astymachus japonicus* Howard, *Scelioencyrtus* species was found to be new and described as *S. mymaricoides* (1960 MS.). There were a few more specimens which could not be easily separated from *Scelioencyrtus* was later identified as a species of *Xanthoencyrtus*. Further studies have since been made and the new species has been described as *Xanthoencyrtus comperei*.

Xanthoencyrtus comperei, New Species

Female

Length 1·17 mm. to 1·23 mm. Colour: body yellow, margin of the metathorax brown, club dark brown, eyes deep red.

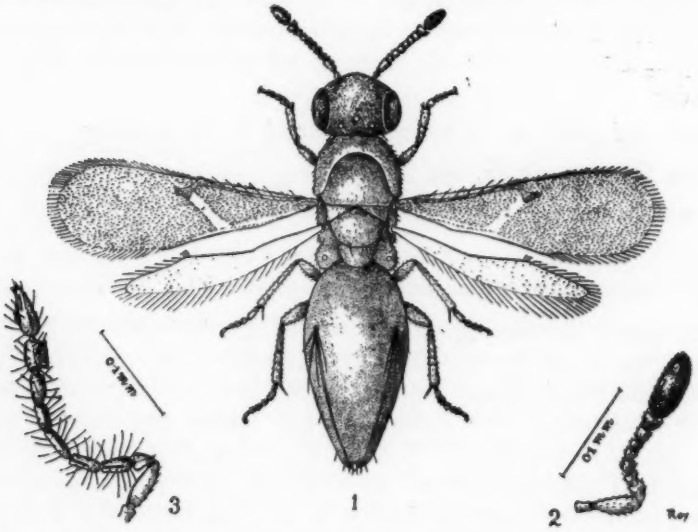
Head rounded, a little wider than thorax, about one and a quarter times longer than wide. Eyes elongate oval, slightly pubescent, inner orbital margins parallel. Cheeks slightly less than half the length of the eyes. Mandibles bidentate, orange yellow. Spines present on the entire surface of the head though stouter and longer on the vertex. Ocelli red, arranged in an obtuse-angled triangle. Interocular space two and a quarter times the interocellar, latter is twice that of the front ocellar and one and one-thirds of ocellocular. Antennæ inserted slightly above the clypeal margin, the sockets about two times their own diameter apart.

Flagellar segments somewhat cup-shaped. The relative lengths of the different components of the antenna are as follows: Scape 14, pedicel 8, flagellar segments 4, 3, 4, 3, 4, 5 and club 20. Antennæ slightly pubescent. Club distinctly two-jointed, the first being about half as long as the second. Sensorial pits are present on the club as well as on the last four flagellar segments though the pits are not so prominent on the flagellar segments.

Thorax.—Finely reticulated though barely perceptible. Hair present all over the thorax but more dense on the prothorax and the part of the mesoscutum. Scutum dome-shaped, larger than the scutellum, one and a quarter times as wide as its median length. Scutellum about one and a half times wider than its median length. Axillae broad, with their inner ends acute and meeting medially. Their scutellar margins somewhat arcuate. A pair of spines are present on the basal margin of the scutum and a pair in the middle of the scutellum. Metathorax narrow. Propodeum very short in the middle region but much longer at the sides. Spiracles circular, situated about one and one-fourth of its diameter away from the side.

Abdomen about one and half times longer than thorax, oblong ovate. Segmentation not very distinct but appears to be six-segmented. Base of abdomen broadly sessile. The vibrissal plates situated a little before the middle of the abdomen with four vibrissæ, of which three are quite long and conspicuous. Paratergites well defined but very narrow. Ovipositor very short, situated at the apex, scarcely protruding.

Wings broad, fully developed and reaching slightly beyond the apex of abdomen. Marginal fringe short. Forewings three times longer than broad. Speculum narrow and oblique, starts from the stigmal downwards and is slightly interrupted in about its middle, then from the lower border



FIGS. 1–3. Fig. 1. *Xanthoencyrtus comperei*, new species. Fig. 2. Antenna, female. Fig. 3. Antenna, male.

continues towards the proximad. Submarginal vein about half the length of the wing. Marginal vein thick but punctiform. Stigmal vein twice the length of marginal. Hind wings seven and half times longer than wide. Cilia on the upper margin very short but on the lower margin three-fourths its greatest width.

Legs.—Typical of the genus. Middle legs comparatively weak and slender, hind legs stouter. Hind tibia equal in length to its femur and trochanter combined. The first two tarsi are equal in length to the next three tarsi.

Male

Length, about 0·94 mm. A little deeper in colour than in female. Essentially similar to the female differing chiefly in antenna and abdomen. Head slightly shorter. Cheeks a little over half the length of the eyes. Antenna broad, the relative lengths of the antennal components are as follows: 25, 10, 15, 15, 12, 12, 15, 28. Flagellar segments cylindrical and each with a long sensorial ridge. The last flagellar segment a little wider than the rest and has a row of about seven funnel-shaped sensorial organs arranged along the longitudinal axis. Long whorls of hairs are prominent on flagellar segments. Club solid, ovate and is as wide as the last funicle.

Described from six females and six males. This species has been named after Dr. Harold Compere of Citrus Experimental Station, California, who has been a source of inspiration to me in my taxonomic work of the family Encyrtidæ.

Holo and allotypes on slides. Collected by R. B. Kaur.

Type locality: Delhi, India.

Host: *Saccharicoccus sacchari* (Cockll.)

Types deposited in the "National Pusa Collection", Division of Entomology, Indian Agricultural Research Institute, New Delhi.

The genus *Xanthoencyrtus* was erected by Ashmead in 1902. Subsequently Mercet (1918) errected the genus *Pholidoceras*. Apparently though there were no generic differences between the two, Mercet preferred to compare his *Pholidoceras* with *Philoponectroma* Brethes and justified his new genus. Later Timberlake (1919) while describing *X. fullawayi* which had two-segmented club synonymised *Scelioencyrtus* Girault and *Mirastymachus* Ashmead with *Xanthoencyrtus* Ashmead. He also noted that most of his species had three-segmented club, as against the two of Ashmead's genotype. However, he did not discuss the genus *Pholidoceras* and its validity in that

paper. Kryger (1943) described a new species under the genus *Pholodoceras*. He observed that though his species, *i.e.*, *P. jali* had a two-jointed club there were not sufficient generic characters to create a new genus and so he placed it under *Pholidoceras*. It was Ferriere (1953) who actually synonymised *Pholidoceras* Mercet with *Xanthoencyrtus* Ashmead. Alam (1957) in redescribing *Xanthoencyrtus semiaptera* (Mercet) has shown a three-segmented club, though Ferriere had shown only a two-segmented club in his figure. Alam mentions that his specimen was collected from the same locality (Silwood Park) as that of Ferriere. It is evident from the above facts that there is a slight individual variation in the number of segments of the female antenna in *Xanthoencyrtus*. Hence the synonymisation of the two genera is justified. It is also necessary to discuss the generic status of *Scelioencyrtus* Girault in the light of present studies. Timberlake (1919) and again Peck (1951) have synonymised *Scelioencyrtus* with *Xanthoencyrtus*. Unfortunately in all the species that were described under the genus *Scelioencyrtus* no male was described as an allotype. Taking only the female, there is very little to differentiate it as a distinct genus from *Xanthoencyrtus*, though there are some secondary characters like marginal fringe of the wings, etc. However, a number of males reared of the genus *Scelioencyrtus* show a clear and generic difference from that of the males of *Xanthoencyrtus*. On the basis of this antennal structure which is a dominant generic character, we must place Girault's *Scelioencyrtus* as distinct from that of *Xanthoencyrtus*. The generic status of *Scelioencyrtus* has been discussed by Compere, Subba Rao and Kaur (1960).

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BIOLOGY AND MORPHOLOGY OF THE IMMATURE STAGES OF *MICROCTONUS INDICUS*, NEW SPECIES (BRACONIDÆ: HYMENOPTERA) A PARASITE OF *PHYLLOTRETA CRUCIFERÆ* GOEZE (CHRYSOMELLIDÆ: COLEOPTERA)

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I. Introduction

The Braconid subfamily Euphorinæ has not been studied thoroughly so far from the point of view of both taxonomy and biology, but Muesebeck (1936) made an important contribution to our knowledge of this group by revising the Nearctic species. However, nothing is known about the Indian forms as is quite evident from the fact that *Microctonus indicus* n.sp., to be described elsewhere, is the first record of the genus *Microctonus* Wesmael, 1853, from the Indian sub-continent.

M. indicus n.sp., like the other known species of the genus, parasitises the adults of a coleopteron: in this case *Phyllotreta cruciferæ* Goeze, a pest of various cruciferous vegetables in Delhi and other parts of India.

From the available literature it is seen that so far only four species of the Euphorinæ have been studied as regards their biology. They are: *Microctonus melanopus* Ruthe (Speyer, 1925); *M. vittatæ* Muesebeck (Smith, 1952); *Perilitus coccinellæ* Schrank (Ogloblin, 1913; Balduf, 1926) and *P. rutilus* Nees (Jackson, 1928). Of these, the studies by Smith (1952) are indeed noteworthy, being detailed and accurate. The biology of *Microctonus indicus*, however, yielded certain points of interest, as would be clear from the following account.

II. Material and Methods

The parasites were first observed in the field in the middle of April, 1958, in rather small numbers because of high temperature. The entire work was carried out with field collected hosts, which were dissected daily. The immature stages thus obtained were either mounted in a glycerine-water medium to study their structure—especially the tracheal system—or preserved

in Kahle's fluid, which proved to be a very good preservative, for permanent mounts.

Attempts to produce a parasite-free laboratory culture of the host were not successful because of the adverse seasonal conditions prevailing in April–May, during which period these investigations were carried out.

III. The Behaviour of the Adult Parasite

The behaviour of the parasite with particular reference to oviposition was studied under the binocular microscope by confining a freshly emerged female with a single host in paired petri-dishes. The female parasite evinced a keen interest in the host by vibrating its antennae and moving briskly. The parasite, however, did not actually insert its ovipositor into the host as long as the latter remained motionless at one place: all the while the female remained in a state of 'tension' just behind the host, its abdomen and ovipositor thrust forward between its legs and projecting beyond and under the head, and the antennæ vibrating. As soon as the host showed signs of moving off, the ovipositor was thrust still forward and rapid jabs made on the thoracic sterna. The ovipositor was kept inserted only for a short while—about 5 seconds—and rapidly withdrawn. Quite often the parasite was frustrated in its attempts by the host actively repelling the attack by means of its legs or by turning round.

IV. Sex Ratio

In comparison with *M. vittatæ* Mues., studied by Smith (1952), a fair proportion of males occur in *M. indicus*. Four males were obtained out of a total number of 40 parasites collected in the field. Furthermore, these males were observed to copulate and were normal in all characters.

V. Parasitism in the Field

Host material, collected from the field during April, 1958, and dissected in the laboratory, revealed that the percentage of parasitism was about 38% during that period.

Table I

	10 batches of field collected hosts, with 10 hosts in each batch									
	1	2	3	4	5	6	7	8	9	10
Number of host found parasitised on dissection	3	5	3	4	2	4	5	3	5	4

VI. SUPER-PARASITISM

Super-parasitism appears to be of common occurrence in this species. A maximum of 3 grubs (2 first instar and 1 early second instar) and 1 egg have been seen in a single host, while the presence of 2 first instar larvæ in one adult beetle was quite frequent.

VII. DESCRIPTION OF IMMATURE STAGES

The egg.—The ripe ovarian egg of this species (Fig. 1) conforms to the usual shape of a braconid egg. The cephalic end is bluntly rounded and the caudal end bears a stalk. The dorsal aspect is uniformly convex while the ventral is somewhat concave. The egg is 0·10–0·115 mm. long, this length including the 0·04 mm. long pedicel. The egg appears somewhat translucent.

First-instar larva.—A first-instar larva soon after hatching is shown in Fig. 2. In side view, the body is slightly curved; the head somewhat bent downward is brownish, sclerotised, while the rest of the body, composed of 13 segments, is more or less transparent.

The head capsule of the first-instar larva is illustrated in Fig. 3. It is distinctly broader than long; the mandibles (*m*) are long, attenuated, deeply sclerotised—of a darker brown colour than the rest of the headcapsule—their tips crossing each other when retracted. The antenna (*a*) are situated anterior to the mandibles and the lobe-like maxillæ (*mx*) are immediately lateral to the mandibles.

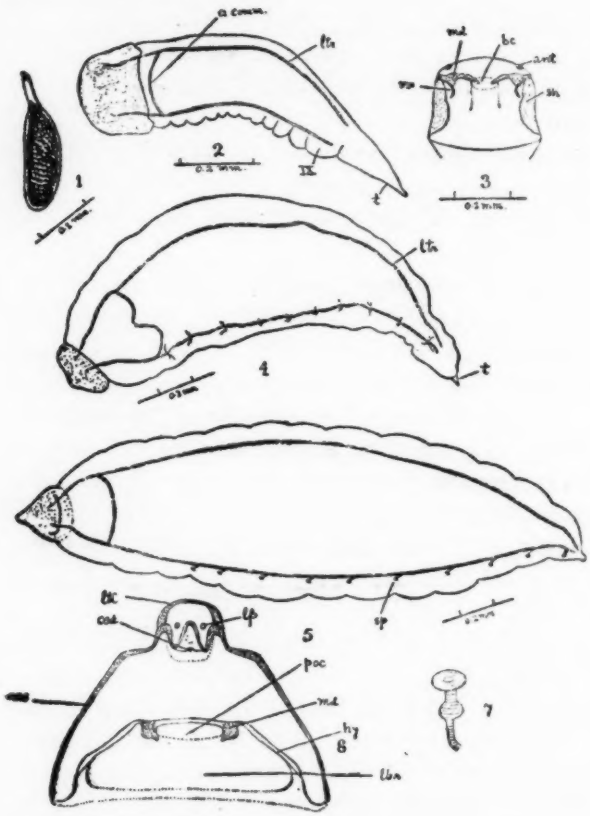
The three thoracic segments are well defined; the first eight adbominal segments are of about equal size, the ninth segment appreciably larger than the preceding one and enlarged on the dorsal aspect because of the globular rectum, while the 10th segment is very much elongated and ends in the 'tail' (*t*).

The tracheal system comprises a pair of thin lateral longitudinal trunks (*ll t*) running from the head and extending into the 9th abdominal segment. A dorsal transverse commissure unites the two lateral longitudinal trunks posterior to the head, but there is no caudal commissure.

As the first-instar larva grows, its appearance is considerably altered from that described above, inasmuch as the gut gets filled with fat globules which impart a yellowish hue to the body. The ' tail ' bends down markedly, and progressively gets shortened. The measurements of the full-grown first-instar larva are: length 0·602–0·606 mm.; width 0·09–0·10 mm.; width of head capsule 0·132–0·134 mm.; ' tail ' 0·07–0·08 mm.

The second-instar larva.—The second-instar larva (Fig. 4) is somewhat rounded and the segmentation is not so distinct as in the first-instar larva.

The head, in comparison with the rest of the body, is smaller in size than in the first-instar and has lost the sclerotic mandibles and 'shields' of that instar. The body contents are more or less obscured by the fat globules which began appearing in the late first-instar; and the 'tail' is now very much reduced and directed ventrally.



FIGS. 1–7. Fig. 1. Ovarian egg. Fig. 2. First-instar larva: Dorsal view (segmentation shown only on the left side). Fig. 3. Head of first-instar larva: dorsal view. Fig. 4. Second-instar larva: dorsal view. Fig. 5. Mature larva: dorsal view. Fig. 6. Head of mature larva: diagrammatic and much enlarged showing the sclerites. Fig. 7. Abdominal spiracle of mature larva: much enlarged. *a.comm.*, anterior commissure. *ant.*, antennal rudiments; *bc*, bucca; *hy*, hypostoma; *lbr*, labrum; *lp*, rudiment of labial palpi; *lsc*, labial stipital sclerome; *ltr*, lateral longitudinal trunk; *mas*, maxillary sclerome; *md*, rudiments of mandible; *mx*, rudiments of maxilla; *osd*, opening of the silk duct; *poc*, preoral cavity; *sh*, sclerotised head shield; *sp*, spiracle.; *t*, tail; IX, IX abdominal segment of first-instar larva.

The tracheal system is well developed in this instar but still not functional inasmuch as the spiracles have not yet been formed. On each side of the body is a lateral longitudinal trunk which extends clearly into the head giving off some short branches; posteriorly the longitudinal trunk runs almost the entire length of the larva, giving off one dorsal and one ventral branch in each of the 10 abdominal segments.

The measurements of the second-instar larva are: length 1·52–1·60 mm.; width 0·30–0·31 mm.; width of the head capsule 0·198–0·201 mm.; 'tail' 0·03–0·04 mm.

The third-instar larva.—The third-instar larva (Fig. 5) is the mature larva which emerges out of the host body. It is cylindrical, with cephalic and caudal ends tapering. The head in this instar (Fig. 6) is well developed and sclerotised.

The prothorax is the largest and the meta-thorax the smallest segment of the thorax. The abdomen is distinctly 10-segmented, the 3rd segment being somewhat larger than the others.

The tracheal system is essentially the same as that of the second-instar larva excepting that the spiracles are present in this instar (*i.e.*, the 3rd) and the tracheal system is, therefore, functional. The structure of an abdominal spiracle, its atrium and part of the tracheal trunk is illustrated in Fig. 7. The measurements of the third-instar larva are: length 2·00–2·22 mm.; width 0·399–0·612 mm.; width of head capsule 0·206–0·218 mm.

VIII. Mode of Issuance

The full-fed last instar larva comes out of the host-body in the manner described below:

The egg is deposited in the body in the body cavity in the thoracic region and the larva on hatching out travels down to the abdomen, where—as observed during dissections—the various immature stages are to be found. The larva continues its journey posteriorwards till, when finally mature, it comes to lodge in the peri-anal region. At the time of issuance, the peri-anal tissues are torn through by the pressure exerted by the mature larva, which comes out of the posterior extremity of the host-body, giving the appearance of having made the exit through the rectum if the whole process is not carefully observed.

IX. Development Period

The parasite was observed to take 10–12 days to complete its development within the host and 3–4 days for pupation outside at room temperature

varying from 86° to 96° F. and 60% R.H. The first-instar period lasted for 5–6 days, the second-instar for 3–4 days and the third-instar for about 2 days at the above temperature and humidity.

X. Discussion

Microctonus indicus, new species, is a parasite of adults of *Phyllotreta cruciferæ* Goeze, a pest of various vegetables in Delhi and other parts of India.

The manner in which the female parasite attacks the host has been described and it seems to agree with the description of the process as given by Smith (1952) in the case of *M. vittatæ* and by workers on the other species of *Microctonus*. It also agrees with *M. vittatæ* in that the region selected for oviposition is invariably the thorax. But in this species, the parasite eggs have been found in any of the three thoracic segments whereas in *M. vittatæ*, according to Smith (1952), the anterior part of the prothorax is most often selected. Also, as in *M. vittatæ*, superparasitism was of common occurrence, at least in the field-collected host material. The maximum number of parasites found in one host was 4 (3 grubs and 1 egg) of which only 1 (an early first-instar larva) was alive, the others having apparently been killed either in combat or by the toxic secretions of the 'survivor'.

In our opinion, there are only three instars in the immature stages of this parasite, *viz.*, the first-instar, the second-instar and the third-instar larva. Smith (1952), however, described as many as 5 instars in *M. vittatæ*. As far as we could gather from a careful perusal of his work, what he regards as third-instar is really the advanced second-instar and his fourth-instar is an early form of the third-instar larva. Further, Smith (*t.c.*: 328) himself, while commenting on the inapplicability of Dyar's law to the immature stages of *M. vittatæ*, makes the following remark: "It is obvious that for the species Dyar's principle fails to apply, whether the correct number of instars present be 4 or 5."

In the occurrence of males, *M. indicus* n.sp. resembles *M. pusillæ* Mues. and *M. morimi* Ferr. and differs from *M. vittatæ*. In the last-named species, Smith (1952) obtained only 1 male out of 612 imagines during two consecutive years, and remarked that reproduction in M. *vittatæ* seemed to be entirely thelyotokus. Now, in *M. indicus* the males were morphologically quite normal and copulation was observed frequently, both in the field and in the laboratory. We, therefore, believe that the males of *M. indicus* are fully functional.

The general structure of the head of the mature larva of *M. indicus* agrees closely with that of *M. vittatæ* though differing in the relative proportions of

the components (Fig. 6). In this species, too, the tentorial hypostome (*hy*) is internal and unpigmented: actually it is of a very light colour as compared to the other chitinous parts, *viz.*, the maxillary and the labiostipital scleromes.

By studying the mode of issuance of the mature larva, we are able to confirm the view expressed by Smith (1952) (*t.c.*: 337) that emergence occurs by means of the tearing of the membranous tissues around the anus—between the pygidium and its parallel ventral sclerite, to be more exact. Speyer (1925) stated that the mature larva comes out of the anus and Clausen (1940) in his remarks on the process of emergence in the Euphorinæ (*t.c.* : 33) stated that the larva cuts a hole through the thin membrane between two abdominal segments. However, Clausen (*t.c.* : 33) also made the following statement: "In some instances it has been reported that the emergence is through the anal opening, but it was more probably through an incision in the membrane nearby." The latter remark of Clausen's is quite correct except for the term ' incision ' since the final instar larva does not have the mandibles fit for cutting the tissues or making ' incisions ' in them. Speyer's view, that the emergence takes place through the anus, seems to have been based on an erroneous observation, for, as explained in the description of the process of issuance in *M. indicus*, the mature larva does give the appearance of making its exit out of the anus if the whole process is not carefully observed.

XI. SUMMARY

Microctonus indicus, new species, is a braconid parasite of the adults of *Phyllotreta cruciferæ* attacking various vegetables in Delhi and other places in India. This is the first record of the genus *Microctonus* Wesmael from the Indian sub-continent. The biology and behaviour of the adult parasite has been briefly described and discussed in this paper and the immature stages have also been described.

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